Weight Maintenance and Food Intake

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Studies on the protein values of diets (Miller and Payne, '61a, b, c) have directed attention to maintenance requirements for nitrogen and energy. In the course of this work a range of diets was constructed and the animals fed these diets were in N equilibrium at different caloric intakes; unexpectedly with these regimens, weight was also maintained. Calorie requirements for maintenance are normally calculated by summation of allowances for basal metabolism and physical activity (Durnin, '61); in the experiments described, difference in physical activity could not explain the observed difference of caloric intake. In the early experiments rats were used and the difference in caloric intake was about 30% (Fryer et al., '61), but the work with pigs described below shows three to fivefold differences in the caloric intake. The findings are discussed in relation to the calculation of calorie requirements, the prescription of diets for weight control, the concept of basal metabolism and specific dynamic action.

METHODS

Gross energies of food, feces, urine and carcasses were determined by the method of Miller and Payne ('59). Metabolizable energy of the diets fed to rats was calculated from the gross energy (Miller and Payne, '59). Metabolizable energy of the diets fed to the pigs was determined by collection of feces and urine over three-day periods. Losses (less than 10%) in the collection of feces were allowed for on the basis of the recovery of chromium oxide added to the diet.

Calorie balances were carried out on rats using the technique described by Fox et al. ('59). Nine weanling littermate rats were divided into three groups of equal weight. The animals in one group were killed, their carcasses dried at 100° and ground together by passing several times through a domestic mincer. The other groups were fed the test diets for one week and treated in the same way. The difference in energy content of the carcasses of the two test groups and the control group was taken to be stored energy.

Expired CO_2 was measured by drawing air (10 liters/min) through a box made of transparent plastic of 600-liter capacity. The air was dried with sulphuric acid, and CO_2 was collected on soda lime (Carbosorb B.D.H.). The CO_2 collected was taken as the change in weight of the soda lime columns and final sulphuric acid bulbs. The efficiency of absorption (CO_2 and H_2O) of the train was checked by including a final column of KOH pellets whose weight remained constant. The box had a floor area (0.8 × 1.2 m) similar to the animals' ordinary cage, and allowed normal activity and access to food. The animal was kept in the box for periods up to 24 hours.

The net protein utilization (NPU) of the diets was determined by the method of Miller and Bender ('55) as modified by Miller and Payne ('61). Net dietary-protein values which are a measure of the utilizable protein in a diet (Platt and Miller, '59) were calculated as:

$$\text{NDpCals \%} = \text{NPU} \times \text{P}$$

where P is the percentage of energy supplied by protein and was calculated from:

$$\frac{25 \times \text{N \%}}{\text{Metabolizable energy/gm}}$$

Diets. The diets for the high-calorie regimens were fed ad libitum, and made by diluting a stock diet with fat and carbohydrate so as to provide a mixture with

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which the animal maintained weight. The low-calorie regimen consisted of an unmodified stock diet fed in an amount also sufficient to maintain body weight. In this way the protein source was always the same and the regimens differed only in the concentration of protein in the diets and the amount fed. Examples are given in table 1.

EXPERIMENTAL

Rat experiments. An examination of more than 300 assays on about 1,000 diets for NPU, showed 108 groups of four 30-day-old rats whose weight (50 to 60 gm) was constant to ± 1 gm for the 10-day period. The caloric intakes ranged from 105 to 225 Cal./day/ kg of body weight to the power of 0.73, although the amount of net dietary protein/day (P × NPU ×

TABLE 1

Composition of diets

	E	N	M
Stock diet	100	20	10
Starch		30	25
Vitaminized starch		5	5
Glucose		15	25
Fat		15	25
Salts		5	5
Cellulose, powdered[1]		—	5
Potato starch		10	—
Metabolizable energy, Cal./gm	4.1	4.1	4.9
Nitrogen, %	4.1	0.83	0.41
NDpCals, %[2]	11.9	4.0	1.7

[1] Solka Floc, Brown Company, Berlin, New Hampshire.
[2] Determined on rats fed ad libitum.

Cal./day/$kg^{0.73}$) showed only a small standard deviation (SD = 1.23), thus:

NDpCals/day/$kg^{0.73}$ for maintenance = 6.24 ± 0.12 SE

In view of the wide range of caloric intakes, balance experiments were set up in order to explain the maintenance of body weight. An example is given in table 2, of complete calorie balances with similar rats fed high- and low-calorie regimens based on diets E and N.

Caloric intakes are in the ratio of 1:1.3, despite the constancy in live weight. This cannot be explained by differences in digestibility, losses in the urine, nor changes in the energy content of the carcass. In classical procedures for caloric balances heat stored is obtained "by difference;" here, heat emission is calculated in this way. These are in the same ratio as the food intakes and indicate that the food consumed is being metabolized. This is confirmed by similar differences in CO_2 expired.

These findings could be accounted for by differences in the activity of the two groups, but attempts to show this were unsuccessful. The rats were placed in a cage suspended from above, and the number of movements of the cage were counted as electrical impulses produced by a gramophone pick-up connected to the cage. In fact the low-calorie group was shown to be slightly more active, which confirmed visual observation.

Pig experiments. Two 28-day-old weanling pigs were maintained at constant

TABLE 2

Balance data for two groups of three rats over a period of one week

	Low-calorie regimen Calories		High-calorie regimen Calories	
	In	Out	In	Out
Food	299		395	
Feces		49		25
Urine		9		12
Carcass loss	47		4	
Heat emitted		288		362
Total	346	346	399	399
Digestibility	83%		94%	
CO_2 expired	86 gm		126 gm	
Activity	670		600	

weight for periods of 40 days with regimens based on diets E and M. Weight curves are shown in figure 1 together with the weight curve of a normal pig. At the end of 40 days the regimens were reversed and this procedure was continued for 6 months. In this way each pig served as its own control. During each period the pig fed the high-calorie regimen consumed 47,500 Cal. and the pig fed the low-calorie regimen only 9,750 Cal., showing a difference of more than 37,000 Cal. equivalent to at least 4 kg of fat. The body weight of the pigs during the first period remained at 4.5 kg. Measurements were made of the energy content of the feces and urine and a typical example is shown in table 3.

Measurement of the activity of these animals presented some problem. The pig on the low-calorie regimen appeared to be more active, but it was considered that as this animal was hungry its activity might

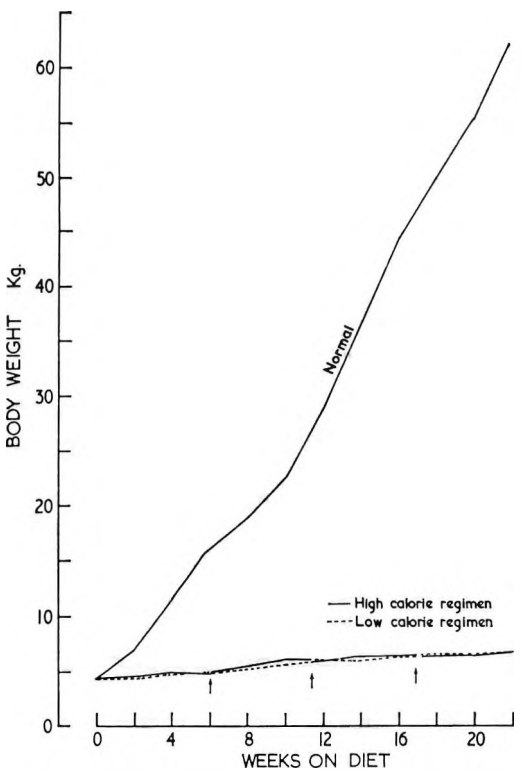

Fig. 1 The weight curves of pigs fed high- and low-calorie regimens compared with the weight curve of a normal pig. The arrows indicate the times when the regimens were reversed.

TABLE 3

Daily caloric intakes of pigs fed high- and low-calorie regimens

	Low-calorie regimen	High-calorie regimen
Gross energy, Cal.	243	1185
% in feces	14.0	4.4
% in urine	4.5	1.7
Metabolizable energy, Cal.	198	1113

be related to the presence of the observer. A time-lapse cine film was therefore taken of the two pigs side by side over a period of 24 hours and this demonstrated that there was very little difference in behavior.

It was not possible to measure expired CO_2 of the pigs described above due to lack of a suitable apparatus. This was constructed later and two other pigs, maintained at constant weight by feeding similar diets at a number of different caloric intakes, were used for this purpose. The data obtained are illustrated in figure 2 and demonstrate that the major part of the food energy was used for metabolic purposes and not lost by some route which had been overlooked.

DISCUSSION

Weight maintenance depends upon the canceling of the gains and losses of a number of body components, the more important of which are fat, protein, water, and minerals. Regimens likely to produce no changes in body weight therefore do not depend solely on caloric intake, but also on the other dietary factors, particularly protein. However since the caloric intake is known to influence the protein value of diets, these factors must be considered together.

Miller and Payne ('61a) have shown that diets having NDpCals 4% met the protein requirements for maintenance of animals fed ad libitum, and Brody ('45) states that the caloric requirements for maintenance is 140 Cal./day/kg of body weight to the power of 0.73. The present work takes into account the interrelationship of caloric intake and NDpCals %, and indicates that maintenance can in fact be achieved over a range of values given by:

$$\text{NDpCals } \% \times \text{caloric intake} = 624.$$

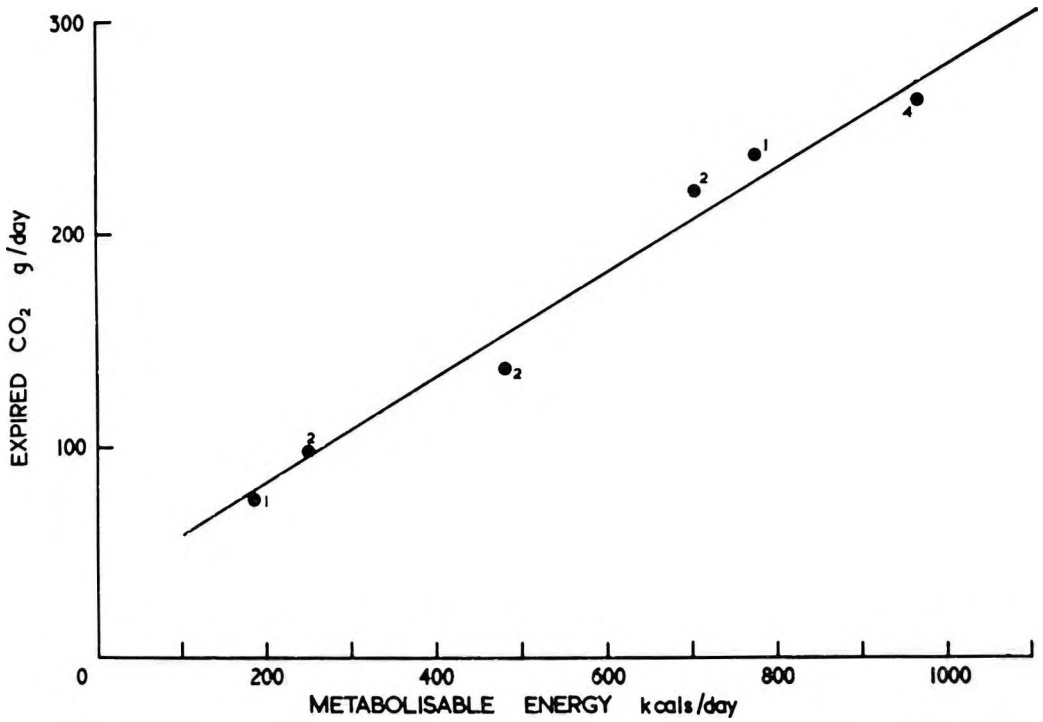

Fig. 2 The relationship between expired carbon dioxide and metabolizable energy. The numerals show the number of independent trials.

This equation is shown in figure 3 drawn on the diagram given by Miller and Payne ('61c); the upper curve encloses all possible conditions with respect to calories and protein in a regimen. This area is divided by the maintenance curve into two regions one of which contains all possible regimens that will support growth.

Also shown on the diagram are lines giving average ad libitum food intakes for the rat and the pig. These are only approximate but indicate a large species variation. The slope of these lines indicates that laboratory animals tend to eat more of diets of higher protein value. For man, Dole et al. ('53) showed that ad libitum food intakes of hospital patients also increased with the concentration of protein in the diet. A study of the literature shows that the pig has an exceptionally high food intake and that other species including man are similar to the rat. The pig was therefore a useful animal for this study.

The two pairs of pigs described in this paper were kept at constant weight for periods of 40 days. During this time the food intakes on the low- and high-calorie regimens were, respectively, 2.4 and 11.8 kg and the animals consumed all the food offered. The difference between these figures is too great to be accounted for by errors due to weighing or spillage, and is calorically equivalent to an amount of fat of the same weight as the pigs. Errors due to variations between individual animals were eliminated by using each animal as its own control. The balance figures show that the food was being absorbed and the recovery of expired CO_2 shows that the absorbed calories were being metabolized.

The animals were kept in cages of 1.0×1.5 m which limited their movements to a little more than lying and standing. Whereas basal metabolism is said to depend upon body weight to the power of 0.73, the energy cost of activity, from a consideration of simple mechanics is related *directly* to body weight. Thus the proportion of energy requirements used for activity with large animals is greater than that used by small animals. For ex-

ample, the energy cost of walking 20 km as a percentage of basal requirements would be 30% for a man, but only 6% for a rat. If we take the figure given by Brody ('45) of 0.5 Cal./kg/meter as the net energy of walking, the difference between the caloric intakes of the two pigs would correspond to a daily walk of 450 km or some equivalent activity, e.g., continuously jumping to a height of 25 cm, 100 times/min. These calculations should be considered together with the measurements of activity.

Finally, difference in the specific dynamic action of the diets tends to make the difference in metabolizable energy greater in view of the higher protein content of the low-calorie diet. Consideration of energy requirements for synthetic processes (Butler, '46; Miller and Payne, '61c), or stored energy in the form of energy-rich phosphate bonds (Wilkie, '60), indicates that these are of several orders of magnitude too small to account for the results of these experiments.

It is currently accepted that the metabolizable energy of food consumed can only be accounted for by (a) changes in weight; (b) changes in body composition; or (c) physical activity. The evidence presented eliminates any of these possibilities and the apparent paradox can be explained only if it is assumed that the animal converts some of the food energy directly to heat. It is important to consider whether the heat involved could be dissipated by the animal, i.e., by radiation, convection, or ventilation of the lungs. There was no difference in body temperature between the two animals and attempts to measure skin temperature were unsatisfactory and are not reported. The pig metabolizing 1,100 Cal. must lose heat at the rate of 50 watts; radiation alone could account

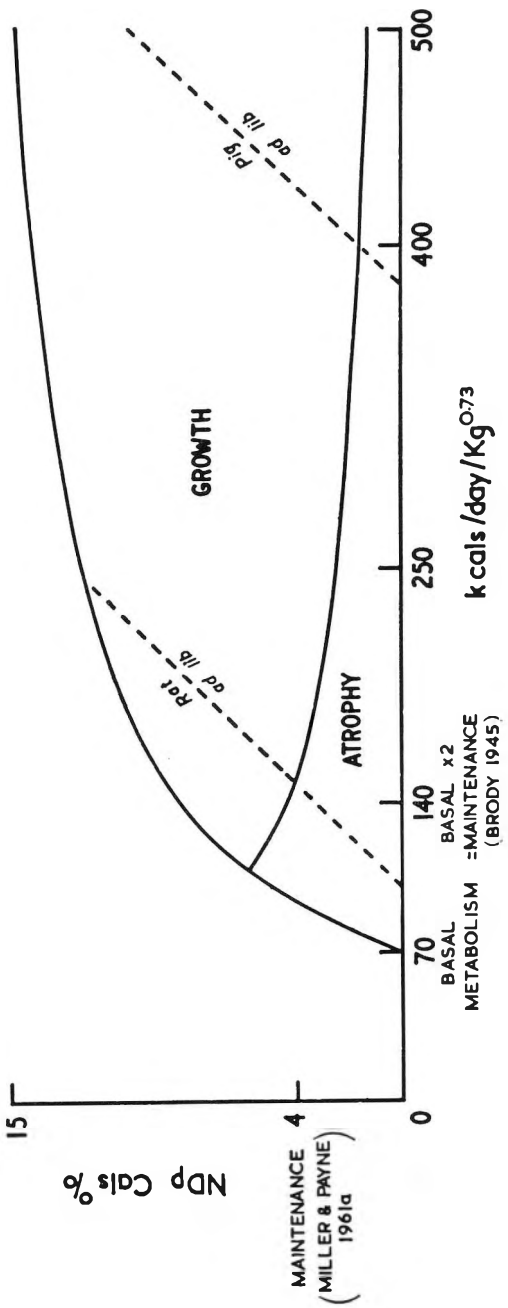

Fig. 3 Diagram showing the region of conditions with respect to protein and calorie supplies where growth is possible.

The upper curve is given by:

$$\text{NDpCals \% (restricted)} = 17\left(1 - \frac{70}{C}\right)$$

and applies when protein synthesis is limited by food energy (Miller and Payne, '61c).

The lower curve is given by:

$$\text{NDpCals \% (maintenance)} = \frac{624}{C}$$

Where C = caloric intake/day/kg$^{0.73}$

for most of this loss. Taking the surface area of the pig at 0.37 m^2, a skin temperature of 37°C and room temperature of 20°C, would result in radiation loss equivalent to 800 Cal./day. The pig on the low-calorie regimen presumably conserves heat by normal processes.

Workers in Heidelberg at the turn of the century suggested that excessive food intakes could be converted directly to heat. Neumann ('02) showed that his own body weight was constant for periods of one year, at daily caloric intakes of either 1,766, 2,199, or 2,403 Cal. He proposed that the excess, or *luxuskonsumption* was directly oxidized, and dissipated as heat. Grafe and Graham ('11) fed a 20-kg dog a diet containing about 20% of protein at caloric intakes of 1,120 and 2,580 Cal./day, and observed weight maintenance on each of these regimens. The American physiologist, Gulik ('22), contrasted "the brilliant successes that science has won in the study of nitrogen equilibrium" with the "rather discouraging inconclusiveness in the work that has been done on .... the balance between the total intake and total output of potential and kinetic energy." His experiments on himself, were for a period of 370 days and were similar to those of Neumann except that the range of food intakes was greater (1,874 to 4,113 Cal./day), and that he kept a meticulous account of his physical activity. On the other hand, Passmore et al. ('55), while drawing attention to the commonplace observation that "some people keep in good health, appear to have a good appetite and yet remain very thin," could find no evidence during short-term experiments, that excess food could be oxidized. Their "thin men" during 10 to 14-day periods consumed either 2,100 or 3,900 Cal./day, and gained weight on the latter regimen. It appears unlikely, however, that the observed rate of gain would have continued indefinitely as, (a) it is equivalent to 65 kg/year, and (b) the work of both Neumann and Gulik shows that transitory weight changes may occur initially in response to changes of regimen. Studies of the food intakes of individuals show frequently that two people of the same sex, age and occupation have widely differing caloric intakes; for example, Widdowson ('47) found that at every age between one and 18 years it was possible to find one child in a group of 20 of the same sex who was eating nearly twice as much as another. More recently, Rose and Williams ('61), in studies with 6 pairs of large and small eaters whose food intakes ranged from 1,600 to 7,400 Cal./day, showed that the weights of all subjects varied little over a period of some weeks despite similar levels of activity. The experiments mentioned above differ from those described in this paper in that the subjects were adult, and that similar foods were consumed in different quantities.

It is accepted (Kleiber and Rogers, '61; Smith and Hoijer, '62) that metabolic body temperature regulation can occur without shivering in animals adapted to the cold, and there is a similarity between this process and the phenomenon described here. It is of interest to consider possible mechanisms for this effect. Biochemical reactions involve small changes in entropy and under conditions of dynamic equilibrium it appears possible that the complex reversible reactions that take place would require a supply of free energy to maintain them. Thus the maintenance of labile stores of some body constituents (e.g., liver glycogen or depot fat), in equilibrium with metabolic pools could contribute to calorie requirements, and the cost of this might be dependent upon the size of the store. If the latter is increased by dietary means the heat output would also increase, and this system would tend to stabilize fat stores and body weight. Kennedy ('61) has suggested that fat stores also control appetite and body activity, but the regulation of heat output is the simplest mechanism for weight control as regulation of either appetite or activity is subject to interference by external factors such as conscious control. But these conjectures require further investigations.

The direct production of heat from fat and carbohydrate would interfere seriously with the evaluation of the specific dynamic action of proteins in mixed diets. Such determinations should also take into account the proportion of the protein that is used for energy purposes dependent upon

the protein: calorie ratio and caloric intake (Miller and Payne, '61a, c).

The results of the experiments described in this paper emphasize the importance of evaluating dietary regimens as a whole, i.e., considering the interrelationships of the nutrients rather than evaluating each component separately. It is difficult, for example, to understand why the reducing diets normally prescribed contain a high proportion of protein. In a previous communication (Miller and Payne, '61c), it was shown that under conditions of caloric restriction much of the protein is used for energy purposes. In theory, a 70-kg man eating less than 1,500 Cal. is unable to retain food nitrogen. Furthermore, the work of Dole et al. ('53) indicates that appetite is stimulated by increasing the protein content of the diet, a factor that makes it more difficult to follow a low-calorie regimen. These authors (Dole et al., '54) successfully treated obese patients with a low-protein, calorically unrestricted diet. Finally, it is clear from our experiments that very high caloric intakes of food low in protein do not necessarily result in weight gains.

Similarly, recommended nutrient allowances should take into account the interrelationship between energy and protein. The two pigs in the experiment were maintained at constant body weight by providing either 15 gm of protein and 243 Cal. or, 7.4 gm of protein and 1,185 Cal./day; the rats, however, showed a smaller range. The common practice of doubling the energy requirements for basal metabolism to obtain the requirement for maintenance, may not be theoretically justifiable, but for most species excluding the pig it is a good approximation. Thus an average maintenance regimen is a caloric intake of 150 Cal./day/$kg^{0.73}$ of a diet of 4% NDpCals.

SUMMARY

1. It was shown that weight maintenance can be achieved in rats over a wide range of caloric intakes. Regimens for this purpose are given by:

$$C \times \text{NDpCals } \% = 624 \pm 12$$

where C = caloric intake/day/$kg^{0.73}$ and NDpCals % = net dietary-protein Cals %.

2. Two pigs were maintained at constant weight with diets providing, respectively, 243 and 1,180 Cal./day. After 40 days the regimens were reversed and body weight was maintained for a similar period.

3. The difference in caloric intakes could not be accounted for by differences in digestibility, losses in the urine, storage of depot fat, nor physical activity.

4. Differences in expired carbon dioxide showed that all the ingested food was being metabolized.

5. It was concluded that food energy may be converted directly into heat.

6. The implications of these observations were discussed in relation to the measurement of the specific dynamic action of proteins, the prescription of diets for weight control, and the allowances of protein and energy to meet requirements.

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Effect of Dietary Regimen on Cessation of Intestinal Absorption of Large Molecules (Closure) in the Neonatal Pig and Lamb[1]

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Piglets, like ruminants and horses, are able to absorb gamma globulin for approximately the first 36 hours of their lives (Brambell, '58). While investigating the selectivity of this absorption phenomenon (Lecce et al., '61), using polyvinylpyrrolidone (PVP) as a nonprotein, high molecular weight, testing molecule, it was observed that piglets nursing the sow appeared to stop absorbing PVP sooner than piglets fed cow's milk. This observation coupled with large variations in time (ranging between 24 to 72 hours) reported by different investigators (Hansen and Phillips, '47; McCarthy and McDougall, '53; Nordbring and Olsson, '58a, b; Deutsch and Smith, '57; Speer et al., '59) for the termination of absorption prompted the present study.

In this study, we were interested in determing whether the cut-off or closure time for absorption can be either lengthened or shortened by various feeding regimens. Most of the earlier attempts that were directed to manipulating this phase with the use of proteolytic inhibitors, hormones, and inhibitors of gastric digestive activity seemed to be ineffectual (Deutsch and Smith, '57; Nordbring and Olsson, '58a, b). Closure time is defined as the age of the pig (in hours) after which he is unable to absorb PVP from his gut. The PVP is considered a suitable marker for testing intestinal absorption since it has molecular weight and osmotic properties similar to those of serum proteins. Furthermore, since the marker is not protein, the uncertain role of the variation in proteolytic enzyme activity in the gut and its effect on closure need not be considered (Hill, '56; Laskowski et al., '57).

MATERIAL AND METHODS

Experiment 1. Pigs in this experiment were allowed either to nurse the sow from the time of birth (nursing pigs) or denied access to any food via the oral route (starved pigs). Random selection determined the pig's treatment. In the nursing group there were 42 pigs, and in the starved group 28. These pigs came from 8 litters. Five of these litters were common to both groups.

The nursing pigs were removed from the sow after predetermined periods of time, given the test dose of PVP via stomach tube, placed in a cage, and 6 hours later bled from the anterior vena cava. The nursing intervals and the number of pigs in each time period were as follows: 4 to 12 hours, 10 pigs; 16 to 20 hours, 7 pigs; 24 to 28 hours, 13 pigs; 36 to 48 hours, 9 pigs; 58 to 96 hours, 3 pigs.

Pigs in the starved group were placed at birth in individual stainless steel cages in an isolation unit. Every 6 hours they were injected intraperitoneally with 10 ml of 5% dextrose in 0.9% saline. One hour before administering the test dose of PVP, they were given 10 ml of 25% dextrose in saline intraperitoneally. Within 6 hours of birth and at 24-hour intervals, they received 50,000 units of procaine penicillin G and 62.5 mg of dihydrostreptomycin intramuscularly. These pigs were given the test dose of PVP at predetermined time intervals and bled 6 hours later from the anterior vena cava. The number of pigs in each time period was as follows: 36 to 48

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hours, 11 pigs; 52 to 60 hours, 11 pigs; 62 to 86 hours, 6 pigs.

Experiment 2. The pigs in this experiment came from two litters. At the time of birth, piglets were placed in individual stainless steel cages in an isolation unit. Here they were fed predetermined amounts of cow's colostrum in several aliquots. The volumes of cow's colostrum fed were 80 ml, 200 to 300 ml, 400 to 500 ml, and 750 ml. The 80-ml group was fed via stomach tube in two 40-ml doses separated by two hours. The other groups were fed portions of 100 to 150 ml in a shallow pan every 4 hours for the first 20 hours of their lives. At 20 hours, all food was removed; at 24 hours, the test dose of PVP was administered via stomach tube. Six hours later pigs were bled from the anterior vena cava. Five pigs (except for 6 in the 400- to 500-ml group) were randomly assigned to each volume group.

In addition, three pigs that had eaten 750 ml of cow's colostrum in 20 hours were then denied food until 48 hours (28 hours since last colostrum feeding). At 48 hours they were given again a test dose of PVP and bled 6 hours later. Also a 17-day-old pig was given a total of 25 gm of PVP within a 2-hour period and bled 12 hours later.

Experiment 3. Lambs were used to determine whether ruminants were similar to piglets in absorption and closure activity. Lambs were managed similarly to piglets except treatment volumes were increased proportionally to the increase in lamb's weight; i.e., lambs were fed approximately 4 times more cow's colostrum, given 4 times more PVP, antibiotic, supportive fluids, and the other treatments.

In addition, ancillary evidence for absorption was obtained by determining with immunoelectrophoresis the absorption fate of fed avian egg proteins and bovine proteins.

Six lambs with the following histories were used: lamb 15, starved for 24 hours, then given 80 ml of 21% PVP; at 30 hours, 80 ml of whole egg were administered; at 36 hours, commenced feeding cow's colostrum.

Lambs 11 and 17, starved for 48 hours, given PVP, commenced feeding cow's colostrum at 54 hours for a total of approximately 1,200 ml until 68 hours of age.

Lambs 12 and 13 (twins) from the time of birth until 20 hours were fed via stomach tube as much cow's colostrum as they could hold. Lamb 12 received 1,245 ml and lamb 13 received 1,560 ml. At 24 hours both lambs were given a test dose of PVP, followed at 30 hours with 280 ml of whole egg. They were starved for the next 24 hours and then given PVP again (54 hours old).

The ewe nursed lamb 14 for 5 days after which the lamb was given no food for 48 hours (water, ad libitum). At the end of the starvation period (seventh day of life), the lamb was given a test dose of PVP.

Closure testing system. At the appropriate time period, 20 ml of 21% PVP was administered to piglets or 80 ml of PVP to lambs through a stomach tube. Six hours later (and often 12 hours with the lambs) 10 ml of blood were removed. The serum from this clotted blood was used to determine whether PVP had been absorbed.

Before attempting to detect PVP in the serum, the serum was first deproteinized, then concentrated fivefold. This was done by diluting the serum 1:3 with water, heating to 100°C for 10 minutes and adding a few drops of 0.1 N HCl to yield maximum precipitation. The clear supernatant containing the PVP (and a small amount of protein migrating as alpha globulin) was dried and reconstituted to one-fifth its original volume in distilled water.

To detect the PVP, 0.004 ml of the concentrated supernatant was subjected to electrophoresis in agar gel. After electrophoresis the PVP was precipitated in the gel by immersing the slides in 16% tannic acid. The gel was washed free of excess tannic acid with running water, dried and stained with Benzyl Red B R.[2] On such a slide PVP shows up as a sharp band migrating in the position of fast gamma globulin (Lecce et al., '61). Measured amounts of PVP placed in serum and treated as above indicated that as little as 2.5 mg PVP/ml serum could be detected on the slide.

[2] Ciba Company, Inc., Charlotte, North Carolina.

RESULTS

Experiment 1. Inspection of the results as shown in the lower part of figure 1 indicates that in pigs nursing the sow, absorption of PVP proceeds without incident for the first 20 hours. Beginning at the 24- to 28-hour period, however, a change in the absorbing capacity takes place. This time period is consonant with the one reported by Speer et al. ('59) who found no effective absorption of homologous antibody beyond 24 hours.

A comparison of closure time in the nursing pigs with the starved pigs (upper part of fig. 1) shows a divergence in time. In the starved pigs, absorption of PVP took place in piglets 52 to 60 hours old and even in a few of the piglets that managed to live to the 62- to 86-hour period. These results are consonant with those of Nordbring and Olsson ('58a) who reported effective absorption of homologous antibody in unfed piglets 72 hours old.

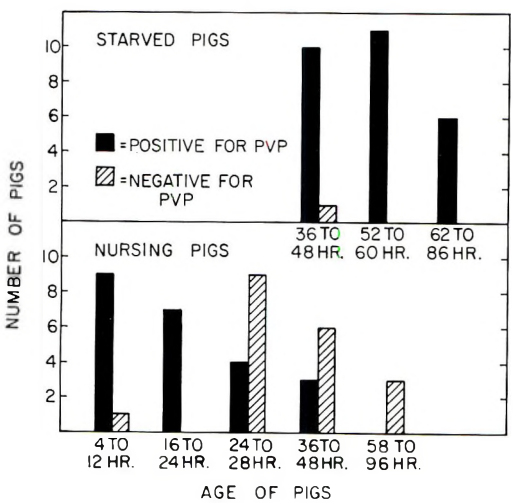

Fig. 1 The effect of nursing and starving on the absorption of polyvinylpyrrolidone (PVP) from the neonatal pig gut.

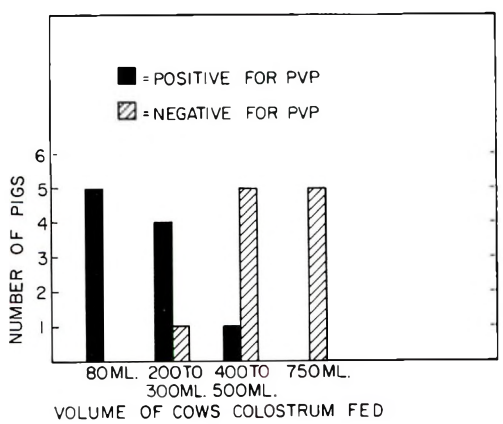

Fig. 2 The effect of feeding cow's colostrum on the absorption of polyvinylpyrrolidone (PVP) from the gut of 24-hour-old pigs.

Experiment 2. The results of experiment 1 indicated that closure was related to feeding rather than age of the animal. Experiment 2 was designed to test this contention.

In figure 2, it can be seen at 24 hours (when unfed pigs can still absorb) that the amount of food the piglet has received will determine whether or not PVP will be absorbed. Under our experimental conditions, it took between 300 to 400 ml of cow's colostrum to give rise to closure. These results harmonized the differences in the reported closure of 24 hours (Speer et al., '59) and the one of 72 hours (Nordbring and Olsson, '58a).

To help rule out the possibility that closure within the first 24 hours may result from some sort of mechanical blockade by the ingested food, three pigs that had received the 750 ml of cow's colostrum were retested at 48 hours (no food since 20 hours). These pigs were still negative at this time.

Experiment 3. Both lamb 15 (starved for 24 hours) and lambs 11 and 17 (starved for 48 hours) were able to absorb PVP at 24 and 48 hours, respectively. Further evidence was obtained by immunoelectrophoresis that these lambs were permeable to large molecules at time of test. Lamb 15, fed whole egg at 30 hours, was positive to egg proteins in the blood 6 hours later (fig. 4A). When fed cow's colostrum at 36 hours, the gut was still permeable to large molecules since bovine colostral proteins were detected in the blood. Likewise, lambs 11 and 17 not only were positive for PVP at 48 hours, but in addition the bovine colostral proteins fed at 54 hours were detected in their blood (fig. 3A). Figures 4B and 3B show that the lambs were immunoelectrophoretically negative when tested for egg and bovine

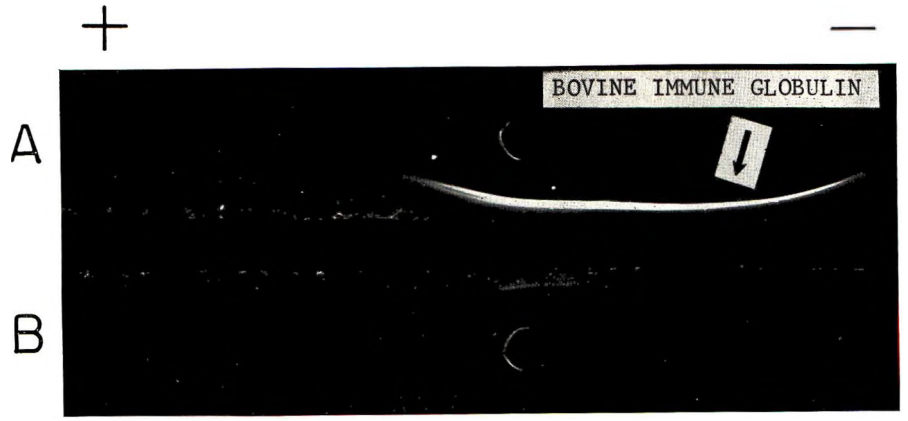

Fig. 3 Immunoelectrophoresis for the detection of bovine whey proteins in the blood serum of lambs fed bovine colostrum when 54 hours old. A — top hole, lamb 17 six hours after feeding bovine colostrum; B — bottom hole, before feeding bovine colostrum; center trough, anti-bovine whey proteins.

Fig. 4 Immunoelectrophoresis for the detection of egg proteins in the blood serum of lambs fed avian eggs when 30 hours old. A — top hole, lamb 15 six hours after feeding eggs; B — bottom hole, before feeding eggs; center trough, anti-egg white serum.

proteins immediately preceding the feeding of these foreign proteins.

Lambs 12 and 13, which were fed as much colostrum as they could hold, did not appear to absorb PVP at 24 hours nor egg proteins at 30 hours. When PVP was readministered at 54 hours (no food for the preceding 24 hours), their blood was still negative for PVP.

Also, we were unable to detect PVP in the lamb that nursed for 5 days and was starved for two days before administering the test dose of PVP, nor was PVP detected in the 17-day-old piglet that had received 25 gm of PVP.

DISCUSSION

Within the limitations of the testing system, our results show that the ability of the piglet and lamb to absorb large molecules does not depend upon the age of the animal but on whether the animal was fed and the amount of food it consumed. Piglets nursing their sow had shortened closure times in comparison to starved pigs. Piglets fed 300 to 400 ml of cow's colostrum behaved similarly to the nursing piglets. In the lamb, feeding likewise affected profoundly the time of closure as evidenced by the absorption in starved lambs of PVP at 48 hours and

bovine colostral proteins at 54 hours. Conversely, in lambs fed approximately 1,500 ml of cow's colostrum, there was neither indication of absorption of PVP at 24 hours nor egg proteins at 30 hours. Furthermore, the lack of absorption did not seem to result from simple blockade by ingested food, since we were unable to reopen (by subsequent starvation) those piglets and lambs already closed.

Confirmation of our results can be found by experiments that are already a part of the literature. For example, Speer et al. ('59), using as a criterion of closure the capacity of piglets to absorb homologous antibody, reported no effective absorption beyond 24 hours of the pig's life. And, these pigs were nursing the sow. Nordbring and Olsson ('58a) using the same criterion reported absorption as long as 72 hours. However, these piglets had been denied food. Recently, Payne and Marsh ('62), tracing the transfer of fluorescent gamma globulin from the lumen to the intestinal epithelium, observed an extreme difference in the capacity of intestinal epithelium to take up gamma globulin depending on whether or not the piglet had been fed.

An obvious implication of our data is to point out the importance of feeding in evaluating a study of absorption, and also to reconcile apparently divergent results already reported. Perhaps another implication may be gleaned by comparing these results with those obtained from a study dealing with *Escherichia coli* in colostrum-free piglets raised in isolation (Lecce and Reep, '62). Here, it appeared that gut permeability and the resistance of the neonatal piglets to diarrhea and bacteremia was paralleled. Perhaps one of the benefits of colostrum (in addition to the well-documented role as an antibody carrier) is that it engenders rapid closure. Then this "nonpermeable" gut, like other mature guts, is resistant to invasion by normal flora microorganisms.

SUMMARY

Piglets that nursed the sow were compared with piglets denied food from birth (starved pigs) with respect to their capacity to absorb large molecules from the gut. The testing molecule was polyvinylpyrrolidone (PVP). Nursing piglets lost their capacity to absorb PVP when approximately 24 to 36 hours old, whereas starved pigs still were able to absorb PVP when 86 hours old (oldest pigs tested). It was possible to mimic nursing piglets and produce closure in 24 hours by feeding piglets 300 to 400 ml of cow's colostrum.

Similar results were obstained with neonatal lambs in that lambs denied food for 24 and 48 hours were permeable to PVP, bovine proteins and egg proteins at these times. And, lambs fed approximately 1.5 liters of cow's colostrum were unable to absorb PVP and egg proteins when 24 hours old. A 5-day-old nursing lamb denied food for the next two days also did not absorb PVP at 7 days.

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Chemical Pathology of Acute Amino Acid Deficiencies

V. COMPARISON OF MORPHOLOGIC AND BIOCHEMICAL CHANGES IN YOUNG RATS FED PROTEIN-FREE OR THREONINE-FREE DIETS[1]

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It has been reported that young rats force-fed purified diets devoid of certain essential amino acids[3] (Adamstone and Spector, '50; Spector and Adamstone, '50; Van Pilsum et al., '57; Sidransky and Farber, '58a, b; Sidransky and Baba, '60), develop pathologic changes that closely resemble many of those found in infants with Kwashiorkor (Trowell et al., '54). In addition, similar changes have been observed in rats force-fed diets containing poor-quality plant proteins (Sidransky, '60). These various findings raised the question whether comparable changes would develop in animals fed a diet entirely free of amino acids or proteins. We therefore studied the morphologic and selected biochemical changes in young rats force-fed a purified diet devoid of protein or amino acids and compared these findings with those in animals force-fed a threonine-free or complete diet. The results were also compared with those obtained in similar animals fed the same diets ad libitum.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the breeding colony of the National Institutes of Health. The animals were maintained with a commerical chow[4] until fed the special diets. The animals were on the average 7 weeks old and weighed 125 gm at the beginning of the experiments. In each experiment several groups of rats were used. Rats were kept in individual wire cages with raised bottoms in an air-conditioned room maintained at 78°F. All animals had free access to water.

The protein-free diet (Rechcigl et al., '57) was composed of the following constituents: (in per cent) dextrose, 15; dextrin, 60.8; vegetable fat,[5] 14, corn oil,[6] 2, cellulose,[7] 2, salt mixture,[8] 4, and vitamin-dextrose mixture,[9] 2.2. The vitamin-dextrose mixture contributed the following number of milligrams of vitamins to each 100 gm of diet: vitamin A concentrate, 9.9 (1,980 units); vitamin D concentrate, 0.6 (240 units); α-tocopherol, 11; ascorbic acid, 99; inositol, 11; choline chloride, 165; menadione, 5; *p*-aminobenzoic acid, 11; niacin, 9.9; riboflavin, 2.2; pyridoxine hydrochloride, 2.2; thiamine hydrochloride, 2.2; calcium pantothenate, 6.6; biotin, 0.04; folic acid, 0.2 and cyanocobalamin (vitamin B_{12}), 0.003. The salt mixture was that described by Jones and Foster ('42). The complete amino acid diet contained 9.2% of essential amino acids and 8.1% of nonessential amino acids substituted for 17.3% of dextrin of the protein-free diet. Essential amino acids were provided in the following percentages: L-lysine hydrochloride, 1.24; L-arginine hydrochloride, 0.75; DL-tryptophan, 0.20;

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[3] Samuels, L. T., H. C. Goldthorpe and T. F. Dougherty 1951 Metabolic effects of specific amino acid deficiencies. Federation Proc., *10:* 393 (abstract).

[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[5] Crisco, Procter and Gamble, Cincinnati, Ohio.

[6] Mazola, Corn Products Refining Company, New York.

[7] Solka Floc, Brown Company, Boston, Massachusetts.

[8] Jones Foster Salt Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[9] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

DL-phenylalanine, 0.90; DL-leucine, 1.60; DL-isoleucine, 1.00; DL-threonine, 1.00; DL-valine, 1.40; DL-methionine, 0.60; and L-histidine hydrochloride, 0.54. The non-essential amino acids were provided in the following percentages: L-glutamic acid, 2.00; DL-serine, 0.50; glycine, 0.70; L-tyrosine, 1.40; L-cystine, 0.20; L-proline, 0.90; DL-aspartic acid, 1.22; and DL-alanine, 1.20. The threonine-free diet was the complete amino acid diet devoid of threonine with dextrin substituted for the missing amino acid. The casein diet contained 18% of vitamin-free casein[10] substituted for 18% of dextrin of the protein-free diet. Each diet was blended with water so that each milliliter contained 0.5 gm of diet. The resulting mixture was in a suitable form for administration by stomach tube.

In the force-feeding experiments animals were divided into 6 groups according to the amount (arbitrarily set at low (7 gm per rat per day) and at high (10 gm per rat per day) and type of diet: (1) low complete (control); (2) low protein-free; (3) low threonine-free; (4) high complete (control); (5) high protein-free; and (6) high threonine-devoid. Force-feeding was performed according to the method of Shay and Gruenstein ('46) using plastic tubes. The rats were force-fed daily at 9 AM, 1 PM, and 4:30 PM. All rats were force-fed 15 ml of casein diet for one day prior to the start of the experiments. Thereafter, the rats fed the low amounts of diet received 12 ml of diet the first day, 14 ml the second and 16 ml the third; whereas the rats fed the high amounts of diet received 18 ml of diet the first day, 20 ml the second and 22 ml the third. On the morning of the fourth day the rats were decapitated approximately 18 hours after the last feeding.

In the ad libitum experiments the animals were divided into three groups according to diet: complete (control), protein-free, and threonine-free. The rats were fed the casein diet for one day before beginning the experimental diets. Rats had available at all times dry diet mixture until the fourth morning when they were decapitated.

Rats were weighed at the beginning and end of each experiment. The organs were weighed fresh. In paired organs, the right organ was weighed. Pieces of tissue from selected organs were fixed in Zenker-formol solution and in 10% formalin. Paraffin sections were routinely stained with hematoxylin and eosin, and some were also stained with Best's carmine and by the periodic acid-Schiff method. Frozen sections of liver after formalin fixation were stained with oil red O.

Collection of material

Liver. After weighing the whole liver, the following pieces were taken: two, one from the median and one from the left lateral lobes, were placed in 30% KOH for glycogen determination; one piece was placed in 50 volumes of ice-cold distilled water and blended in a Waring blender for two minutes; suitable aliquots of this suspension were used for catalase and for nitrogen determinations; a large piece was frozen at − 15°C for subsequent lipid determination.

Pancreas. The whole pancreas was weighed after removing surrounding adipose tissue and lymph nodes. Part of the organ was homogenized in ice-cold 0.02 M phosphate buffer, pH 7.6, and suitable aliquots were taken for nitrogen determinations. The remainder was stored at − 15°C.

Gastrocnemius muscle. The right gastrocnemius muscle was removed, weighed, and then stored at − 15°C. Following thawing the tissue was added to distilled water and blended in a Waring blender. A suitable aliquot was taken for nitrogen determination.

Kidney. The right kidney was removed, weighed, and then blended in cold distilled water with a Waring blender. Suitable aliquots were used for catalase and for nitrogen determinations.

Chemical analyses

Tissue nitrogen. This was measured by determination of Kjeldahl nitrogen (Perrin, '53) on aliquots of the liver, pancreas, gastrocnemius muscle, and kidney.

Liver lipid. The frozen aliquot was thawed, ground to a dry powder with anhydrous sodium sulfate and extracted with chloroform for 24 hours. After eva-

[10] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

poration of the chloroform, the residue was extracted with petroleum ether and the lipid remaining on evaporation of this solvent was weighed.

Pancreatic enzymes. Aliquots of pancreatic homogenates were analyzed for amylase activity by the method of Smith and Roe ('49) and for trypsin activity by method of Schwert and Takenaka ('55). For the latter determination, the enzyme was activated by enterokinase[11] (Chernick et al., '48).

Catalase. The enzyme activity was determined on 50 μliters of homogenates, using the modification (Greenfield and Price, '56) of the spectrophotometric method of Beers and Sizer ('52). A continuously recording spectrophotometer (Cary Model No. 11 MS) with a log-absorbance attachment permitted accurate and rapid assays using a relatively low concentration of substrate.

Liver glycogen. Approximately 1-gm aliquots of liver were used to determine glycogen by the method of Seifter et al. ('50).

RESULTS

In a preliminary experiment 4 groups of rats were force-fed different amounts of protein-free diet for three days. Four rats were used in each group. The diet intake in grams of diet per rat per day for rats in group 1 was 13.0; group 2, 9.8; group 3, 6.9; and group 4, 3.4. A fifth group was allowed to eat the diet ad libitum for three days and these animals consumed on the average 6.3 gm of diet per rat per day. Three of 4 rats in group 1 and one of four rats in group 2 died during the three-day feeding period. Due to the high mortality in group 1, and, since the animals in group 4 consumed less diet than the animals eating ad libitum in group 5, it was decided to force-feed the protein-free diet in two amounts, 7 and 10 gm/rat per day, in the later experiments. In the subsequent three-day force-feeding experiments 16 of 35 rats receiving 10 gm diet/day and one of 26 rats receiving 7 gm diet/day died during the feeding period. Most of these deaths occurred within the first day of feeding and at autopsy no gross findings were observed that could be used to explain the deaths.

Force-feeding experiments

The body and organ weights of each group of animals in the force-feeding experiments are summarized in table 1. Among the groups of animals that received 7 gm of diet per rat per day, the animals receiving the protein-free diet lost the most weight, the animals fed the threonine-free diet lost somewhat less, and the control animals lost the least weight. The mean wet weight of the liver in animals force-fed the threonine-free diet was significantly increased, whereas that of animals force-fed the protein-free diet was decreased in comparison with that of the controls. The pancreas and kidney weighed less in the animals fed the protein-free diet than did the same organs in animals supplied with the control or threonine-free diets. The gastrocnemius muscle and spleen of the control animals weighed more than did the same organs of the other two groups. The testis and adrenal gland weighed about the same in all three groups.

Among the groups of animals that received 10 gm of diet per rat per day, the control animals gained weight, whereas the other two groups lost weight. The livers of animals fed the threonine-free diet weighed more than those of the controls, whereas the livers of animals fed the protein-free diet weighed less than those of the controls. The pancreas and kidney weights of animals supplied with the control and threonine-free diets were similar but each of these organs was significantly heavier than the same organ in the animals fed the protein-free diets. The gastrocnemius muscle and spleen of animals receiving the threonine-free diet weighed less than those organs in animals fed the other two diets. The testis was somewhat heavier in the control animals than in the experimental animals. The adrenal gland weighed less in the protein-free group than in the other two groups. These results are similar to those described for the groups of animals ingesting less total diet (7 gm of diet per rat per day). However, the differences are more accentuated in the groups of animals

[11] Enterokinase, Pentex Biochemicals, Inc., Kankakee, Illinois.

TABLE 1

Change in body and organ weights of rats fed low or high amounts of control, protein-free or threonine-free diets

Group	No. rats	Diet intake	Body weight			Liver	Pancreas	Gastrocnemius muscle	Kidney	Testis	Spleen	Adrenal
			Onset	Sacrifice	Change							
		gm/rat/day	*gm*	*gm*	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Force-feeding experiments												
Control	9	7	126	124	−2	4.42 ± 0.14[1]	537 ± 35[1]	684 ± 25[1]	536 ± 13[1]	911 ± 33[1]	477 ± 26[1]	17 ± 1[1]
Protein-free	29	7	125	116	−9	4.08 ± 0.07[2]	491 ± 11	654 ± 17	466 ± 7[3]	897 ± 26	400 ± 16[2]	18 ± 7
Threonine-free	9	7	127	121	−6	5.34 ± 0.12[3]	531 ± 17	633 ± 29	565 ± 13	997 ± 36	388 ± 33[2]	20 ± 3
Control	4	10	129	132	+3	5.58 ± 0.20	575 ± 9	663 ± 15	597 ± 14	895 ± 34	454 ± 48	21 ± 3
Protein-free	22	10	128	122	−6	4.69 ± 0.08[3]	510 ± 12[3]	674 ± 14	489 ± 11[3]	830 ± 38	372 ± 24	16 ± 2
Threonine-free	6	10	128	125	−3	6.26 ± 0.17[2]	569 ± 19	631 ± 23	610 ± 10	855 ± 29	304 ± 17[2]	22 ± 3
Ad libitum experiments												
Control	3	11	126	128	+2	5.27 ± 0.21	532 ± 20	673 ± 15	634 ± 37	828 ± 33	668 ± 88	18 ± 1
Protein-free	13	8	128	115	−13	4.75 ± 0.17	448 ± 22[2]	622 ± 53	444 ± 9[3]	804 ± 45	434 ± 55	15 ± 1
Threonine-free	4	6	121	106	−15	4.35 ± 0.16[2]	451 ± 15[2]	565 ± 38[2]	437 ± 23[3]	741 ± 39	467 ± 51	16 ± 1

[1] Mean value ± standard error of the mean.
[2] P between 0 01 and 0.05 (probably significant).
[3] $P < 0.01$ (highly significant).

ingesting more diet than in those ingesting less diet.

In table 2 are shown the amounts of liver lipid, glycogen and nitrogen; kidney nitrogen; pancreatic nitrogen, amylase, and trypsin; and gastrocnemius muscle nitrogen in groups of animals force-fed the different diets. In animals ingesting 7 gm per rat per day, values for the liver lipid, glycogen and nitrogen of rats force-fed the threonine-free diet were greater than in animals receiving the complete or protein-free diets. Kidney nitrogen was decreased in animals fed the protein-free diet. Pancreas nitrogen, amylase and trypsin were moderately decreased in animals fed the protein-free diets. Muscle nitrogen was minimally decreased in rats supplied with the threonine-free diet. In animals ingesting 10 gm per rat per day the liver lipid, glycogen and nitrogen were increased in rats force-fed the threonine-free diet. Kidney nitrogen was low in rats fed the protein-free diet. Pancreatic nitrogen, amylase and trypsin were markedly decreased in rats receiving the protein-free diet. Pancreatic amylase was moderately decreased but trypsin only minimally decreased in rats fed the threonine-devoid diet. Muscle nitrogen was decreased in rats receiving the threonine-free diet. These results indicate that the rats fed the greater amounts of protein-free or threonine-free diets developed somewhat more accentuated changes than did rats supplied with the lesser amounts of diet.

Table 3 summarizes the data on liver and kidney catalase activity of rats force-fed control, protein-free, or threonine-free diets. The results are expressed on a concentration basis (units per gram of organ and units per milligram of nitrogen) and also per total organ (units per organ). In the animals ingesting either 7 or 10 gm/day, liver catalase activity on a concentration basis or per total liver was markedly decreased in both experimental groups of animals in comparison with control animals. The animals fed 10 gm/day of the experimental diets had a greater decrease of liver catalase activity than the animals fed 7 gm/day of the experimental diets. The most marked decrease was in total liver catalase activity in the rats supplied with the protein-free diet. Similar

TABLE 2

Analyses of liver, pancreas and right gastrocnemius muscle of rats fed control, protein-free or threonine-free diets

Group	No. rats	Diet intake	Liver			Kidneys	Pancreas			Right gastrocnemius muscle nitrogen
			Total lipid	Glycogen	Nitrogen	Nitrogen	Nitrogen	Amylase	Trypsin	
		gm/diet/rat/day	*mg/liver*	*mg/liver*	*mg/liver*	*mg/kidneys*	*mg/pancreas*	*units × 10⁻³/pancreas*	*units × 10⁻³/pancreas*	*mg/muscle*
					Force-feeding experiments					
Control	9	7	195 ± 32[1]	76 ± 18[1]	146 ± 4[1]	31.9 ± 3.7[1]	17.4 ± 1.1[1]	12.5 ± 2.6	35.9 ± 3.8	21.8 ± 1.5[1]
Protein-free	29	7	193 ± 8	71 ± 7	136 ± 3	26.9 ± 0.6	15.8 ± 0.5	8.9 ± 0.6	19.8 ± 1.8[3]	24.7 ± 0.9
Threonine-free	9	7	320 ± 37[2]	185 ± 26[3]	162 ± 6[2]	34.4 ± 3.6	18.1 ± 0.6	12.4 ± 0.8	36.5 ± 2.1	19.7 ± 1.2
Control	4	10	355 ± 31	130 ± 48	166 ± 9	33.8 ± 4.1	18.5 ± 4.0	14.5 ± 2.4	39.2 ± 3.5	23.0 ± 0.5
Protein-free	22	10	300 ± 16	94 ± 13	145 ± 3[3]	29.0 ± 0.8	15.9 ± 0.7	7.5 ± 0.6[3]	15.3 ± 1.0[3]	24.7 ± 0.8
Threonine-free	6	10	518 ± 61[2]	213 ± 49	186 ± 12	37.3 ± 3.2	17.1 ± 1.0	8.7 ± 1.0	28.7 ± 1.8[3]	20.1 ± 1.0[3]
					Ad libitum experiments					
Control	3	11	166 ± 47	235 ± 93	155 ± 5	35.7 ± 2.3	18.0 ± 1.0	12.7 ± 0.5	34.5 ± 4.8	21.3 ± 0.5
Protein-free	13	8	203 ± 7	330 ± 53	126 ± 4[3]	27.8 ± 1.4[3]	15.2 ± 0.6[3]	9.1 ± 1.3[3]	20.3 ± 7.9	19.5 ± 1.9
Threonine-free	4	6	166 ± 47	246 ± 28	107 ± 13[2]	28.0 ± 0.0	15.9 ± 0.6	12.6 ± 1.0	34.4 ± 4.2	18.2 ± 0.8[2]

[1] Mean value ± standard error of the mean. [2] P between 0.01 and 0.05 (probably significant). [3] $P < 0.01$ (highly significant).

TABLE 3

Liver and kidney catalase activity of rats fed control, protein-free or threonine-free diets

Group	No. rats	Diet intake	Liver catalase activity			Kidney catalase activity		
		gm/rat/day	*units/gm liver*	*units/mg N*	*units/liver*	*units/gm kidney*	*units/mg N*	*units/kidneys*
			Force-feeding experiments					
Control	9	7	172 ± 13[1]	5.3 ± 0.1[1]	766 ± 64[1]	68.0 ± 2.0[1]	2.3 ± 0.2[1]	70.5 ± 2.7[1]
Protein-free	29	7	132 ± 4[2]	4.0 ± 0.0[2]	539 ± 17[2]	52.6 ± 1.6[2]	1.8 ± 0.1[2]	48.5 ± 1.8[2]
Threonine-free	9	7	117 ± 5[2]	4.0 ± 0.1[2]	619 ± 30	58.5 ± 2.5[2]	1.9 ± 0.1	65.5 ± 4.5
Control	4	10	133 ± 4	4.5 ± 0.1	742 ± 46	54.0 ± 1.0	1.9 ± 0.3	63.5 ± 3.5
Protein-free	22	10	102 ± 4[2]	3.2 ± 0.2[2]	474 ± 16[2]	51.6 ± 2.1	1.8 ± 0.1	50.4 ± 2.3[2]
Threonine-free	6	10	93 ± 5[2]	3.2 ± 0.1[2]	583 ± 34[2]	45.5 ± 0.2[2]	1.5 ± 0.2	55.5 ± 1.5
			Ad libitum experiments					
Control	3	11	155 ± 12	5.3 ± 0.4	810 ± 52	60.0 ± 7.0	2.1 ± 0.3	75.0 ± 5.0
Protein-free	13	8	95 ± 4[2]	3.6 ± 0.1[2]	448 ± 23[2]	54.3 ± 3.8	1.73 ± 0.1	38.7 ± 0.4[2]
Threonine-free	4	6	103 ± 13[3]	4.3 ± 0.4	450 ± 77[3]	60.0[4]	1.9[4]	52.0[4]

[1] Mean value ± standard error of the mean.
[2] $P < 0.01$ (highly significant).
[3] P between 0.01 and 0.05 (probably significant).
[4] Value is based on pooled samples.

but a less marked decrease in catalase activity was present in the kidneys of rats fed the experimental diets.

Ad libitum experiments

The rats fed the complete diet consumed an average of 11 gm per rat per day and gained weight, rats receiving the protein-free diet ate 8 gm per rat per day and lost weight, and rats fed the threonine-free diet ate 6 gm per rat per day and lost weight (table 1). The weights of the liver, pancreas, gastrocnemius muscle, kidney, testis, spleen and adrenal gland of rats fed the protein-free and threonine-free diets were in all cases less than that of the same organs in rats on the control diet (table 1).

Table 2 summarized various analyses performed on the liver, kidney, pancreas and gastrocnemius muscle of rats fed the three different diets. The liver lipid and glycogen were essentially similar in animals fed all diets. On the other hand, liver and also kidney nitrogen were decreased in rats supplied with the protein-free and threonine-free diets. Gastrocnemius muscle nitrogen and pancreatic nitrogen were diminished in the experimental animals. Pancreatic amylase and trypsin activity were decreased in rats fed the protein-free diet.

Table 3 summarizes the liver and kidney catalase activities of rats fed the diets ad libitum. Marked depression of liver and kidney catalase activities was noted in the animals fed the experimental diets in comparison with those supplied with the control diet.

Morphologic changes
Force-feeding experiments

Liver-low diet intake (7 gm per rat per day). On microscopic examination, the livers of rats fed the control or protein-free diets were normal. After hematoxylin and eosin staining the cytoplasm was not vacuolated. The liver cells showed no excess lipid and glycogen when stained for these constituents. The nuclei of hepatic cells contained several small nucleoli. The livers of rats fed the threonine-free diet were similar to the others except that some livers contained a slight increase in lipid

in the periportal areas and also a slight increase in glycogen.

High diet intake (10 gm per rat per day). Histologically, the majority of the livers of rats fed the complete or the protein-free diets were normal. After fat stain, a few livers had a slight increase of periportal lipid. The livers of rats fed the threonine-free diet had a moderate-to-marked increase of lipid and of glycogen in comparison with the controls. The increased lipid was present intracellularly only in the hepatic cells around the portal triads while the glycogen was diffusely distributed. The nuclei of liver cells usually contained a single large nucleolus.

Pancreas. The pancreas was normal in animals fed the low and high complete and low threonine-free diets. In animals fed the low and high protein-free and the high threonine-free diets the pancreases showed changes. In these animals the acinar cells of the pancreas showed a mild decrease in the ratio of cytoplasm to nucleus and the number of zymogen granules was diminished in comparison with controls. The nuclei appeared somewhat crowded together with a disordered arrangement.

Thymus gland and spleen. The thymus gland and spleen were histologically normal in the animals fed all diets except the high protein-free and high threonine-free diets. In most of these animals the organs showed a reduction in the number of lymphocytes. In the thymus the decrease of lymphocytes was present in the cortex so that a sharp demarcation between the cortex and medulla was lost. In the spleen, in addition to the decrease in lymphocytes, there was a prominence of the connective tissue.

The following organs showed no gross or microscopic changes: kidney, adrenal gland, and testis.

Ad libitum experiments

The pancreas of rats fed the protein-free diets showed histologically mild atrophic changes. Other than this, all organs of rats fed the complete, protein-free or threonine-free diet were normal.

DISCUSSION

The results of this study show that in three-day experiments young rats force-fed a diet devoid of protein or amino acids develop different pathologic changes from those in rats force-fed the same diet with added essential and nonessential amino acids but devoid of threonine. Although the rats fed the threonine-free diet developed a periportal fatty liver with increased hepatic glycogen, the rats supplied with the protein-free diet showed no pathologic changes in the liver. The pathologic changes were more marked when the animals received more diet (10 gm per rat per day) than when they received less diet (7 gm per rat per day). When animals were fed the protein-free, threonine-free or complete diets ad libitum, few if any pathologic changes were found.

Although total liver nitrogen decreased in animals force-fed the protein-free diet or fed ad libitum the protein-free or threonine-free diets, and remained unchanged or even increased in animals force-fed the threonine-free diet, liver catalase activity was significantly decreased in all animals fed the experimental diets. The results for kidney catalase activity were similar to the results for liver catalase. Our results in rats fed diets devoid of threonine are in agreement with the results obtained by Van Pilsum et al. ('57) who obtained similar findings with isoleucine or phenylalanine deficiency. In other experiments (Williams et al., '49; Bothwell and Williams, '54; Van Pilsum et al., '57; Sidransky and Farber, '58b) in which animals were force-fed diets deficient in certain single essential amino acids, other liver enzymes, such as xanthine oxidase, succinic oxidase, and choline oxidase were found to have decreased activity even though liver nitrogen remained essentially unchanged. Whether this decrease in liver enzyme activity is due to a decreased rate of synthesis or to an increased rate of breakdown is at present not known. Recently techniques have been developed to measure *in vivo* the kinetics of catalase synthesis and breakdown (Price et al., '61; Rechcigl and Price, '61). The development of similar techniques for other enzymes may be helpful in answering this question.

The results in the present experiments with rats force-fed the threonine-free diet are in agreement with earlier studies

(Sidransky and Farber, '58a, b; Sidransky and Clark, '61). The diet used in the present experiments differed in the composition of the vitamins, minerals, fats, and carbohydrates from that used in the earlier studies. Also the Sprague-Dawley rats used in the present experiments were somewhat older and larger (7 weeks and 125 gm) than those used in the earlier experiments (4 weeks and 75 gm). In another earlier study (Sidransky and Farber, '58c) even larger (130 to 240 gm) and older Sprague-Dawley and Wistar rats were used. However, the results in all of these studies are similar even though the overall composition of the diet and the age and size of the animals varied.

In the present experiments rats were force-fed two different quantities of deficient diets and developed different degrees of pathologic changes depending on the amount of diet consumed. This adds further support to the concept that the total quantity of deficient diet consumed is important in relation to the pathologic changes. In earlier studies with rats fed synthetic diets deficient in certain single essential amino acids (Sidransky and Farber, '58a, b; Sidransky and Baba, '60) and with rats fed diets containing poor-quality plant proteins (Sidransky, '60), it was found that the animals that were force-fed the diet developed many pathologic changes, whereas the animals that ate the same diets ad libitum consumed much less diet and developed few, if any, pathologic changes.

In addition to the importance of the total quantity of deficient diet consumed, the amount of carbohydrate or calories in the deficient diet is also critical (Sidransky and Clark, '61). Our present experiments stress the importance of the presence of amino acids in the deficient diet in inducing pathologic changes, especially in the liver. When amino acids are absent from the diet and are replaced by dextrin, relatively few pathologic lesions develop. This suggests that a complexity of factors is involved in the induction of pathologic changes in animals force-fed a diet deficient in a single essential amino acid. The pathologic changes, therefore, should not be attributed to an essential amino acid deficiency alone, but rather should be attributed to a nutritional imbalance created experimentally by force-feeding a purified diet adequate in all components but deficient in one essential amino acid.

SUMMARY

Young rats force-fed for three days a purified diet devoid of protein or amino acids developed different pathologic changes from those in rats force-fed the same diet with added essential and non-essential amino acids but devoid of threonine. The rats force-fed the protein-free diet showed no pathologic changes in the liver, whereas the rats fed the threonine-free diet developed an increase in liver lipid with a periportal distribution, an increase in liver glycogen, and a slight increase in liver nitrogen content. Similar but less marked changes in the liver were observed in rats fed the same diets at a level of 7 gm rather than 10 gm per rat per day for three days. Liver changes did not develop in any of the rats fed the same diets ad libitum. Liver catalase activity was decreased markedly in livers of rats fed the protein-free and threonine-free diets under both feeding conditions. Pancreatic atrophy along with a decrease in nitrogen, amylase, and trypsin developed in rats force-fed the protein-free and threonine-free diets and in animals fed the protein-free diet ad libitum. The results indicate that in force-feeding experiments the amino acid content of purified diets devoid of threonine is important in the induction of pathologic changes in the liver.

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Effects of Altering the Ratio of Indispensable to Dispensable Amino Acids in Diets for Rats[1,2]

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Shortly after the nutritional indispensability of certain amino acids was recognized, investigators in the field of protein and amino acid nutrition entertained the idea of successfully growing young animals with synthetic diets containing only the indispensable amino acids (Hopkins, '16). Since then several investigators have shown that a diet containing only the indispensable amino acids supports only a slow rate of growth (Kinsey and Grant, '44; Wretlind, '49) and that other sources of nitrogen are required for the attainment of normal or optimal growth rates and normal physiological function.

Much qualitative work has established that diets containing only the indispensable amino acids are improved by additions of individual dispensable amino acids (Womack and Rose, '47), mixtures of dispensable amino acids (Rose et al., '48), and other nonspecific sources of nitrogen (Lardy and Feldott, '49; Rechcigl et al., '57). The work in this field has been reviewed by Frost ('59) and Greenstein and Winitz ('61), and therefore will not be discussed in detail.

Despite the large amount of qualitative work, very few investigations have dealt quantitatively with the relationship between indispensable and dispensable amino acids. Frost and Sandy ('51) showed that when one-third of the total nitrogen in an amino acid diet was supplied by glutamic acid, arginine, or diammonium citrate large growth responses were obtained, and that when one-fifth of the total nitrogen was supplied by glycine, or by alanine and glycine, growth was appreciably stimulated. Frost ('50) stated that "the ratio of essential to other than essential amino acid nitrogen is of primary importance when studying the efficiency of any given source of other than essential amino acid nitrogen." He also stated that the optimal level of nonspecific nitrogen had not been determined accurately.

Recent work in this laboratory has shown that the chick, at least, is quite sensitive to changes in the ratio of indispensable to dispensable amino acids (Stucki and Harper, '61). The use of DL-amino acids in the diets used in that study made precise interpretation difficult, but it appeared that over the dietary nitrogen range of 3.6 to 4.2%, the optimal amount of nitrogen from dispensable amino acids was about 33% of the total. For subsequent studies of the optimal ratio of indispensable to dispensable amino acids the rat was selected as the experimental animal. This made exclusive use of L-amino acids more economical and made it possible to avoid the problem of the fate of nitrogen from D-amino acids. The results of these experiments are reported in this paper.

EXPERIMENTAL

Rats were fed amino acid diets which varied in total nitrogen content and in the ratio of indispensable to dispensable amino acid nitrogen (I/D ratio). The total nitrogen content of the diets was varied from 1.2 to 2.4%, which corresponds to a variation in protein content (N × 6.25) from 7.5 to 15%. The I/D ratio was varied from 0.5 (i.e., 33% of the total N from indispensable amino acids) to infinite (all N from indispensable amino acids). Diets differing in N content and I/D ratio were prepared by using different amounts of two mixtures of amino acids. The indispensable amino acid mixture (table 1) contained the individual amino acids in

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proportions based on the requirements of the rat as determined by Rao et al. ('59). The composition of the dispensable amino acid mixture is shown in table 2.

The quantities of the two amino acid mixtures in the diets used in this experiment are given in table 3. In addition to amino acids all diets contained the following percentages of other dietary components: water-soluble vitamin mixture (Harper, '59), 0.5; mineral mixture (Harper, '59), 5.0; fat-soluble vitamin mixture, 0.5 (supplies 4,000 IU of vitamin A, 2,000 IU of vitamin D, and 100 mg of α-tocopherol/kg of diet); choline chloride, 0.17; corn oil, 5.0; and cornstarch, to make 100%.

The animals were male weanling rats of the Holtzman strain. The individual starting weights of the rats ranged from 42 to 50 gm, but average individual weights for all groups were between 45 and 48 gm. Experimental groups were composed of 5 animals each.

The rats were weighed daily to determine the rate of adaptation to the amino acid diets. Food consumption by groups was also determined daily and any spilled food was collected on papers so that appropriate corrections could be made.

At the end of the experiment, 17 days, the animals were weighed. They were then killed with ether and the gastrointestinal tracts and livers were removed. The livers were weighed immediately after removal. The gastrointestinal tracts were taken for studies of microflora and hence were neglected in the subsequent tissue analyses. The carcasses with liver and gastrointestinal tract removed ("empty carcass") were placed on uniform rectangles of nitrogen-free plastic film[3] and weighed to determine the carcass weights.

The carcasses from each group were pooled, dried at 70°C in a forced-air oven for 24 hours and then at 110°C in another oven for a further 24 hours. The carcasses were weighed again to determine the original moisture content by difference and were then ground in water in a large Waring Blendor. Sulfuric acid was added to the suspensions to make the final acid concentration approximately 5 N in a volume of 2 liters. The acidified suspensions were heated in an autoclave at 120°C for 6 hours and were then diluted to 4 liters. Duplicate aliquots of 10 ml each were taken for determination of nitrogen by the micro-Kjeldahl method using a mercury catalyst.

Samples of 700 mg were taken from each liver and the 5 samples from each group were pooled for subsequent determinations. The pooled sample of liver from each group was ground while still wet with a known amount of anhydrous Na_2SO_4 in a weighed evaporating dish and dried at 65°C for 24 hours. The samples were reground and dried for another 24 hours at 110°C. After dry weight was determined the samples were quantitatively transferred to extraction thimbles and extracted with chloroform for 24 hours. The weight of fat recovered was determined, and the entire extracted samples in the extraction thimbles were subjected to macro-Kjeldahl analysis for determination of liver nitrogen.

Similar carcass and liver analyses were also performed on weanling rats to deter-

TABLE 1

Indispensable amino acid mixture (mixture A)[1]

Amino acid	Relative amount[2]
L-Arginine·HCl	2.18
L-Histidine·HCl·H_2O	2.38
L-Isoleucine	4.90
L-Leucine	6.24
L-Lysine·HCl	10.33
L-Methionine	4.54
L-Phenylalanine	6.52
L-Threonine	4.54
L-Tryptophan	1.00
L-Valine	4.90

[1] Supplies 12.47 gm N/100 gm mixture.
[2] Based on requirements as established by Rao et al. ('59).

TABLE 2

Dispensable amino acid mixture[1] *(mixture B)*

Amino acid	Relative amount
L-Alanine	1.0
L-Aspartic acid	1.0
L-Cystine	1.0
L-Glutamic acid	12.5
Glycine	7.5
L-Proline	1.0
L-Serine	1.0
L-Tyrosine	1.0

[1] Supplies 12.70 gm N/100 gm mixture.

[3] Saran, Dow Chemical Company.

TABLE 3

Composition of amino acid diets (experiment 1)

Nitrogen	Group no.	I/D[1] ratio	Mixture A	Mixture B
% of diet			*gm/kg*	*gm/kg*
1.2	1	infinite	96.2	none
	2	4.0	77.0	18.9
	3	2.0	64.1	31.5
	4	1.0	48.1	47.2
	5	0.5	32.1	63.0
1.5	6	infinite	120.0	none
	7	4.0	96.2	23.6
	8	2.0	80.1	39.4
	9	1.0	60.1	59.1
	10	0.5	40.1	78.7
1.8	11	infinite	144.2	none
	12	4.0	115.6	28.2
	13	2.0	96.2	47.2
	14	1.0	72.2	70.8
	15	0.5	48.1	94.5
2.1	16	infinite	168.2	none
	17	4.0	134.6	33.1
	18	2.0	112.2	55.1
	19	1.0	84.2	82.7
	20	0.5	56.2	110.2
2.4	21	infinite	192.5	none
	22	4.0	154.0	37.8
	23	2.0	128.4	63.0
	24	1.0	96.2	94.5
	25	0.5	64.1	126.0

[1] Indicates ratio of indispensable to dispensable amino acid nitrogen.

mine an average starting composition to be used in calculations of nitrogen gain.

RESULTS

The growth results are presented in figure 1. Growth is reported as total gain from the start of the experiment for several selected days from the 17-day experimental period.

Two groups fed 15% casein diets served as controls. One group (AL) was fed the casein control diet ad libitum and the other (PF) was fed an amount of the casein diet equal to the amount of amino acid diet consumed by the group which had the greatest total gain. The total gains for the casein-fed groups are indicated numerically along the bottom of figure 1.

Several general trends are evident from examination of figure 1:

(1) Nitrogen level and I/D ratio both affected initial rate of gain. The effect of a change in I/D ratio on the initial rate of gain was greatest when the diet was high in nitrogen, and became less marked as the nitrogen content of the diets decreased. For example, the difference between the initial gains of groups 1 and 5 was much less than the difference between the gains of groups 16 and 20.

(2) Except in the series receiving 1.2% of N, the groups fed the diets with the lowest I/D ratio, showed the greatest early gains. Later in the experimental period, however, the rates of gain of the groups fed the diets having the lowest I/D ratio tended to decrease relative to those of groups fed the diets having intermediate I/D ratios. The diet containing 1.2% of N with an I/D ratio of 0.5 (diet 5) provided only enough indispensable amino acids to satisfy about 60% of the requirements of the rat; hence the inferior performance of the group fed this diet was not unexpected. Groups fed the diets having intermediate I/D ratios showed the best final performance, but as nitrogen level increased, the difference between

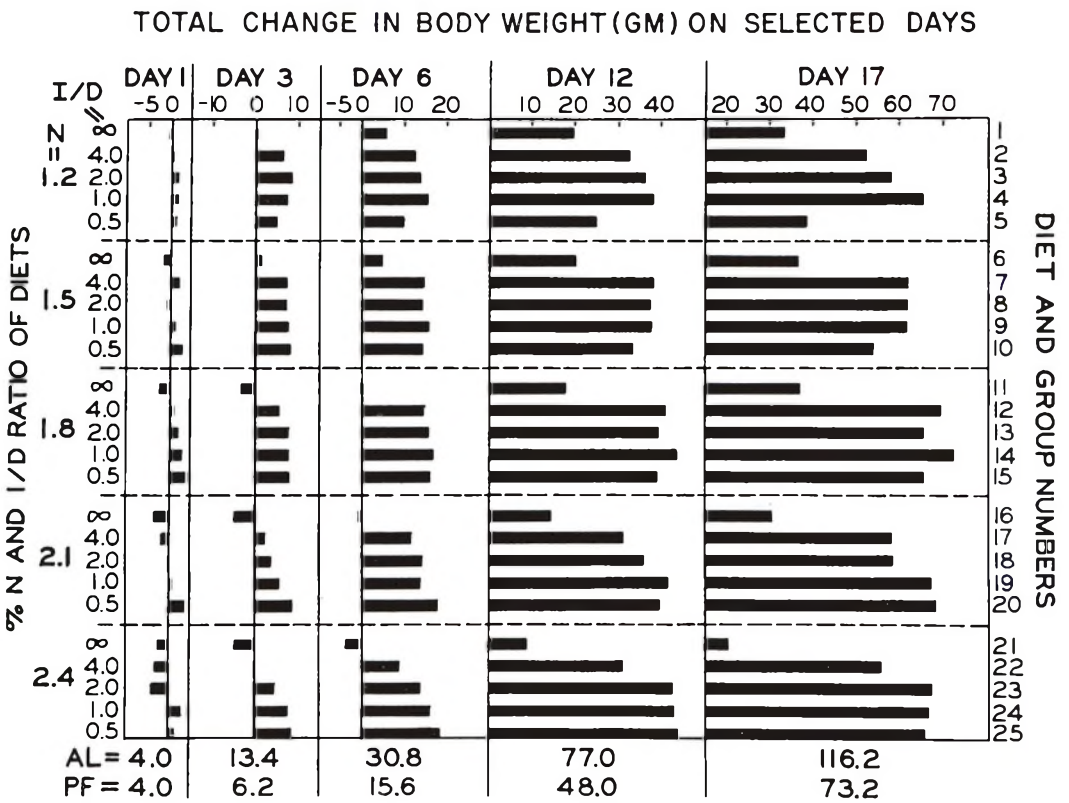

Fig. 1 Effect of nitrogen content and ratio of indispensable to dispensable amino acids (I/D) on weight gain of rats fed protein-free amino acid diets. The bars represent the total gain in grams for each group on the day indicated along the top of the figure. The values along the bottom of the figure indicate the gains of groups fed a casein diet ad libitum (AL) or pair-fed (PF) to the amino acid group gaining most rapidly.

groups fed the diets having intermediate and low I/D ratios decreased. (Compare the 17-day gains of groups 23 and 25 with those of groups 8 and 10).

(3) Groups fed the diets containing only indispensable amino acids (diets 1, 6, 11, 16 and 21) had the lowest rates of growth and the rate tended to fall as the dietary nitrogen content was increased from 1.8% to 2.4%.

(4) When the groups that gained the most weight in the series receiving the highest and the lowest levels of nitrogen are compared, nitrogen level by itself appeared to have little effect on the maximal growth obtainable. However, the superior growth of group 14 suggests that there may be some interaction between nitrogen level and I/D ratio.

Rats fed the casein diet ad libitum (group AL) gained weight much more rapidly than rats fed any of the amino acid diets or the casein diet on a paired-feeding basis. The weight gain of the pair-fed group receiving casein (group PF) was the same as that of the best amino acid group.

Food consumption figures are presented in table 4, along with food efficiency and "protein" (N × 6.25) efficiency ratios.

The pattern of food consumption can be seen from the figures in table 4. Food consumption decreased when the nitrogen content of the diet and the I/D ratio were both high. The depressing effect of a high nitrogen level on food consumption was more marked when the I/D ratio was infinite than when it was 4.0. Also, when

TABLE 4

Food consumption and performance data

Nitrogen level in diet		Ratio of indispensable to dispensable amino acid nitrogen (I/D ratio)				
		Infinite	4.0	2.0	1.0	0.5
%						
	Group no.	1	2	3	4	5
1.2	FC[2]	118	147	155	165	130
	FE[3]	28	36	38	40	30
	PER[4]	3.75	4.76	5.01	5.28	3.95
	Group no.	6	7	8	9	10
1.5	FC	104	144	143	147	150
	FE	35	43	43	42	36
	PER	3.77	4.60	4.62	4.47	3.83
	Group no.	11	12	13	14	15
1.8	FC	93	154	143	159	159
	FE	40	45	46	46	41
	PER	3.54	4.00	4.09	4.05	3.68
	Group no.	16	17	18	19	20
2.1	FC	84	129	139	149	168
	FE	37	46	42	46	41
	PER	2.79	3.47	3.23	3.48	3.12
	Group no.	21	22	23	24	25
2.4	FC	72	117	141	138	141
	FE	28	47	48	48	47
	PER	1.87	3.16	3.18	3.22	3.10

[1] Casein group fed ad libitum: FC, 206; FE, 56; PER, 3.76. Casein group pair-fed: FC, 157; FE, 47; PER, 3.12.
[2] FC indicates total gm food consumed/rat.
[3] FE indicates gm gain in weight/100 gm food consumed.
[4] PER indicates gm gain in weight/gm protein consumed.

the I/D ratio was infinite, although total food consumption decreased as nitrogen level increased, the total amount of nitrogen consumed was nearly the same for each group. The average nitrogen consumption for groups 1, 6, 11, 16 and 21 was 1.63 ± 0.05 gm (mean ± standard error). This situation did not obtain when the I/D ratio was lower, although three of the groups (12, 17 and 22) fed diets with I/D ratios of 4.0 had nearly identical nitrogen intakes.

When the food efficiency ratios for all groups receiving the same dietary level of nitrogen were averaged food efficiency increased as the nitrogen level was raised to 1.8%. Average values for the groups fed the diets containing 1.2, 1.5, 1.8, 2.1 and 2.4% of nitrogen were, respectively, 34, 40, 44, 42 and 44. When values for all groups receiving diets having the same I/D ratio were averaged the values for I/D ratios of infinity, 4.0, 2.0, 1.0 and 0.5 were, respectively, 36, 43, 43, 44, 39. Efficiency of food utilization was greatest for groups fed the diets with intermediate I/D ratios, the pattern resembling that observed for total gain.

Protein efficiency ratios (PER's) calculated on the basis of live-weight gain, were extremely high for groups fed some of the amino acid diets. Three of the groups receiving the lowest dietary level of nitrogen showed the highest PER values (groups 2, 3 and 4). Within each series of nitrogen levels, PER values were depressed at both the highest and lowest I/D ratios. Also, PER values decreased, as would be expected, as the nitrogen content of the diet increased. The PER for the group receiving the casein diet ad libitum (group AL) was intermediate in the range of values observed for all groups, but it was significantly higher than the PER values obtained for the groups receiving amino acid diets containing similar amounts of nitrogen. However, the PER value for the pair-fed casein group (PF) was approximately the same as those ob-

served for the amino acid groups receiving the same amount of nitrogen.

Since body composition of the groups fed the different diets varied somewhat, a more appropriate measure of nitrogen utilization, nitrogen incorporation efficiency (NIE), was devised. This, unlike PER, takes into account the actual nitrogen content of the whole rat, and thus provides a direct measure of protein gain. It is defined as the ratio of grams of nitrogen gained per gram of nitrogen consumed, and is computed by subtracting the nitrogen content of the weanling rat from the nitrogen content of the experimental rat and dividing by the nitrogen consumed during the experimental period. For example, the nitrogen content of a 47-gm weanling rat was 1.06 gm and the average nitrogen content of the rats in group 14 was 2.79 gm; hence the difference was 1.73 gm of nitrogen. The average nitrogen consumed by the rats in group 14 was 2.86 gm, and therefore the value for NIE is $(1.73/2.86) \times 100$ or 60%.

The values for NIE for the experimental groups, expressed as percentages of the theoretical value of 1 gm of nitrogen gained/gm of nitrogen consumed, are presented in table 5. The theoretical value does not take into account the requirement of the growing rat for maintenance. The values for NIE were characteristically high for groups receiving the lowest level of nitrogen and decreased as the nitrogen content of the diets increased. The values either approach a maximum at the central I/D ratio, as in the series receiving the two lowest levels of nitrogen; or form a plateau, as in the series receiving the three highest levels of nitrogen. Extreme values for I/D ratio resulted in low NIE values irrespective of the level of nitrogen.

Since it was readily apparent both from this work and from other work on amino acid diets that young growing animals usually require a period of adaptation to amino acid diets before normal or near-normal growth rates are attained, some experiments were made in an effort to shorten or eliminate the adaptation period. Because group 14 grew best, diets having approximately the same composition as that used for this group were used in further experiments on the adaptation phenomenon. Glutamic acid alone was substituted for the mixture of dispensable amino acids because it was found that omission of the other dispensable amino acids did not result in depression in growth. Cornstarch was replaced by dextrin[4] in all subsequent experiments.

Various treatments were tested for their effects on rate of adaptation; among these were injections of thyroxine, insulin, and saline; and gradual replacement of an isonitrogenous casein diet with the amino acid test diet. None of these treatments increased the rate of adaptation.

The results of the carcass and liver analyses showed no general pattern, but certain trends were evident. Liver fat content appeared to increase, within each nitrogen level, as the I/D ratio decreased,

[4] Moist cornstarch heated for 2 to 3 hours at 115°C in an autoclave then dried and ground.

TABLE 5

Efficiency of nitrogen utilization[1]

Nitrogen level in diet	Ratio of indispensable to dispensable amino acid nitrogen (I/D ratio)				
	Infinite	4.0	2.0	1.0	0.5
%					
1.2	62	66	69	67	55
1.5	61	66	74	65	49
1.8	53	59	60	60	51
2.1	46	50	49	48	41
2.4	37	50	48	51	42

Group AL	Group PF
65	60

[1] Nitrogen incorporation efficiency (NIE) as percentage of theoretical.

but variability within some of the groups was large. Normal amounts of liver fat (3 to 5%) were observed for most groups but groups 15, 20 and 25 had 6, 8 and 6%, respectively. In all of the groups with high values, however, most of the increase was contributed by one or two animals with extremely fatty livers. Liver nitrogen content ranged from 2.4 to nearly 2.9% (15 to 18% crude protein) and showed a tendency to increase with increasing dietary nitrogen.

Carcass protein content tended to be lower when the I/D ratio of the diet was low, but comparison, by nitrogen level, of the groups having diets with the same I/D ratio showed no consistent trend. The range of variation of carcass crude protein extended from 14 to 17%.

DISCUSSION

I/D ratio and amino acid diets. Frost and Sandy ('51) estimated, on the basis of rat repletion studies, that 20 to 30% of the nitrogen supplied in amino acid diets should be "other than essential amino acid nitrogen for maximum repletion." Rosenberg ('59) concluded that approximately equal quantities of nitrogen should be supplied from dispensable and indispensable amino acids. Greenstein et al. ('57) observed the best overall performance in rats fed a diet (no. 26) in which 62% of the nitrogen was "nonessential." The chick was found to be quite sensitive to changes in the I/D ratio of the diet (Stucki and Harper, '61) but this was not true for the rat, as indicated by the similarity of the growth responses and of values for nitrogen utilization of the groups fed diets with intermediate I/D ratios within each of the series receiving different levels of nitrogen. The chick grew best only when the I/D ratio was close to 2.0, but the rat grew well over a range of I/D ratios from 4.0 to less than 1.0. This range includes the I/D ratios of all diets containing proteins and those of the most commonly used amino acid diets.

Animals fed the amino acid diets did not grow as rapidly as those fed the casein diet ad libitum. In the first 6 days of the experiment the average gain of the group fed the casein diet ad libitum was 6 gm/day compared with 3 gm/day for the best of the groups receiving amino acids. In the last 5 days of the experiment, however, the growth rates of the best amino acid groups averaged 6 gm/day compared with not quite 8 gm/day for the group fed the casein diet ad libitum. Nevertheless, the nitrogen and food utilization values indicate that many of the amino acid diets were used very efficiently. Of particular interest is the observation that the pair-fed casein group (PF) and the amino acid group with the greatest growth showed generally the same performance with the single exception of the PER. Since the amino acid diet contained substantially less total nitrogen and since growth and food intake of the two groups were the same, the PER of the casein group would necessarily be lower. This suggests that, at least under conditions of equal food consumption, the two diets are nutritionally equivalent.

The excellent utilization of nitrogen, as evidenced by the PER and NIE values for diets containing the lower levels of nitrogen and having intermediate I/D ratios, indicates that these diets contain the indispensable amino acids in well-balanced proportions. The similarity of performance of group PF and group 14 suggests that inadequate food consumption is the factor limiting the growth of animals fed the best of these amino acid diets and that the major problem remaining in the development of amino acid diets that are as satisfactory as those containing protein would be solved if greater voluntary food consumption could be induced.

The significance of the period of adaptation required by rats fed these diets containing crystalline amino acids is not known. Since in a few experiments using the same diets but different lots of animals no adaptation or lag period was observed, the adaptation period may be more a characteristic of the particular lot of animals used or of some environmental factor not readily discerned than a characteristic of the diets. Hepburn et al. ('60) also noticed that young growing animals required an adaptation period before attaining normal or near-normal growth rates on amino acid diets. On the other hand, Sauberlich ('61) did not observe an adaptation period

in his experiments but he remarked that rapid early growth required the presence of some of the nonessential amino acids. In our experiments the most rapid adaptation was observed in groups receiving the highest percentage of dispensable amino acids but rapidity of adaptation did not appear to be directly related to final performance.. In two experiments in which the diets of Hepburn et al. ('60), Sauberlich ('61) and a diet previously used in our laboratory were compared, all groups showed a lag in growth during the first few days. Only by adding casein to the amino acid diet have we been able to prevent the early lag in the growth of rats fed large amounts of amino acids.[5] Or, looking at this another way, the amount of amino acids included in some of the better amino acid diets does not retard the growth of rats receiving from 8 to 12% of casein.

Nitrogen incorporation efficiency. The measurement of nitrogen incorporation efficiency (NIE) used in these experiments resembles a procedure used by McCollum and Simmonds ('29) for determination of biological value of proteins and the net protein utilization (NPU) procedure of Bender and Miller ('53). Both NIE and NPU are based on carcass analysis but the NIE value does not include a correction for maintenance requirement. The advantage of using a group fed a protein-free diet to indicate the maintenance requirement, as is done in the NPU method, is difficult to assess. It is likely that the maintenance requirement of a protein-deprived animal of different body weight from the test animal is not identical to the maintenance requirement of the healthy growing test animal. In addition, the shortened method of determining NPU (Miller and Bender, '55) assumes that carcass N:H_2O ratios are constant at any given age regardless of the previous dietary treatment. In one experiment Bender and Doell ('57) reported a NPU of 58.5 with 44-day-old rats fed a 10% casein diet. Although the NIE would be expected to be lower than the NPU because the NIE does not contain a correction for maintenance requirement, a NIE of 65 was obtained for a 15% casein diet with rats similar in age to those used in Bender's and Doell's experiment.

Some composite value incorporating several of the parameters measured in nutritional studies is needed to permit assessment of nutritional adequacy or superiority in relation to normal or optimal growth. While NIE may not represent the ideal composite value, it provides a means of detecting deviations in body composition which may be important in the assessment of a diet.

SUMMARY

Male weanling rats were fed a series of amino acid diets in which both the nitrogen content and the ratio of nitrogen from indispensable and dispensable amino acids were varied. Growth, food consumption, and final body composition were determined for all experimental groups.

The nitrogen of several of the amino acid diets was used very efficiently, indicating that the indispensable amino acids were present in well-balanced proportions. The amino acid diets which contained indispensable amino acids only or relatively large excesses of dispensable amino acids were inferior to diets with moderate amounts of both indispensable and dispensable amino acids.

Rats were shown to be much less sensitive to the ratio of indispensable to dispensable amino acid nitrogen (I/D ratio) than are chicks. Growth of rats fed diets having I/D ratios between 4.0 and 1.0, was generally satisfactory, whereas earlier work had shown that chicks grew well only when the I/D ratio was near 2.0.

On the basis of growth performance alone a diet with 1.8% of total nitrogen proved superior to the other amino acid diets. This diet contained equal amounts of nitrogen from both the dispensable and the indispensable amino acids. The performance of a group fed this diet and that of a group pair-fed a casein control diet were similar in all respects except protein efficiency ratio. When nitrogen utilization was used as the criterion of performance, diets containing less than 1.8% of total nitrogen were superior to diets with 1.8% of nitrogen or more.

The term, "nitrogen incorporation efficiency," was introduced to permit direct comparisons of efficiency of utilization of the nitrogen in these diets. This term rep-

[5] Uupublished observations.

resents the actual percentage of ingested nitrogen incorporated into the carcass and is based on body weight, food consumption and carcass nitrogen analysis.

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Tryptophan-niacin Metabolism

I. PREGNANCY, OVARIAN HORMONES AND LEVELS OF TRYPTOPHAN INTAKE AS FACTORS AFFECTING THE TRYPTOPHAN-NIACIN METABOLISM OF THE RAT[1]

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Ovarian hormones play a definite role in the metabolism of nicotinic acid. In the rat, reduction of hormonal activities induced by ovariectomy results in significant decreases in the urinary excretion of the metabolites of nicotinic acid, whereas increases in the concentration of ovarian hormones, whether resulting from pregnancy or from administration of a combination of progesterone and estrogen, produce marked augmentations in the output of niacin metabolites (Lojkin, '56). In the woman, the increases in the excretion of the metabolites of nicotinic acid which occur in late pregnancy are so great that the total output often exceeds the sum total of the niacin consumed per se plus the niacin, which was thought to be available from dietary tryptophan (Lojkin et al., '52). It seemed pertinent to determine whether the increases in the excretion of the metabolites of nicotinic acid, which occur in the pregnant and in the ovarian-hormone-injected animals, are accompanied by any changes in the niacin reserves of the body. Beher et al. ('52) observed that administration of testosterone propionate to rats induced definite changes in the urinary output of N^1-methylnicotinamide (MNA) and the hepatic pyridine nucleotide concentration. Both the levels of excretion of niacin metabolites and the pyridine nucleotide concentration in the blood and organs of animals and human beings are known to be affected by oral or parenteral administrations of tryptophan, niacin and some niacin derivatives. However, the resulting changes in pyridine nucleotide concentration and MNA output do not necessarily proceed simultaneously and are not similar (Vivian et al., '58; Burch et al., '55).

The present paper represents the results of an investgiation of the effects of pregnancy and administration of ovarian hormones on the concentration of hepatic pyridine nucleotides in rats under different dietary conditions. It includes an estimation of minimal tryptophan requirements for pregnancy and a study of tryptophan-niacin metabolism of pregnant and non-pregnant rats fed several niacin-free diets, which differ in their tryptophan content and are equal in their concentration of other essential amino acids and total nitrogen, and also fed one low-protein diet.

EXPERIMENTAL

Rats. In the first two sets of experiments rats of the Wistar strain were used. Further work was performed on rats of the Osborne-Mendel strain, which had less individual variations in the levels of urinary excretion of MNA. Although the total MNA excretion of these animals is higher than that of the Wistar rats, the percentage of increases in the output of MNA resulting from pregnancy are similar in these two strains.[2] Since some individual variations in MNA output were observed even between littermates in both strains, pre-experimental 24-hour urine samples of all the rats were analyzed for MNA and suitable controls were selected for each experimental animal on the basis of matching MNA excretion values.

Diets. The control or breeders' ration consisted of 10% of meat scraps, 60% of

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[1] Contribution 17 of the University of Massachusetts Agricultural Experimental Station.
[2] Guild, L. P., M. E. Lojkin and A. W. Wertz 1954 Strain differences in niacin metabolism. Federation Proc., *13:* 459 (abstract).

whole wheat and 30% of whole milk powder, to which 1.5% of NaCl was added. The composition of the experimental diets is presented in table 1. The nitrogen content of diets A, B, C and D was 3% and the respective concentrations of tryptophan were 0.136, 0.093, 0.068 and 0.014%. The concentrations of all the other essential amino acids were kept constant by varying the content of the tryptophan-free proteins and supplementing diets B, C and D with small amounts of crystalline amino acids. Diet E contained 1.2% of casein as the only source of nitrogen and amino acids. The animals were fed ad libitum. A niacin-free vitamin supplement was given daily.[3]

Experiment 1. Virgin rats were mated and either allowed to continue to consume the breeders' ration or fed an experimental diet from the first day of gestation. Twenty-four hour urine samples were collected prior to and at intervals during pregnancy for MNA determination. On the twentieth day of gestation each pregnant rat and its nonpregnant control were decapitated. Their livers were analyzed for pyridine nucleotide and observations were made on the condition, number and weight of the fetuses.

Experiment 2. Three groups of animals, each consisting of 12 adult female rats, were fed niacin-free, 0.136, 0.093, or 0.068% tryptophan diets, and the fourth group of 12 rats was allowed to continue to consume the breeders' ration. Six rats in each dietary group were injected intramuscularly with a combination of 4 mg of progesterone and 0.5 μg of estrone in sesame oil for 10 consecutive days. On the eleventh day the injected animals and their noninjected controls were killed.

Experiment 3. Adult male rats were fed the experimental niacin-free diets. Twenty-four-hour urine samples were collected prior to and at weekly intervals during the experiment. All animals were killed at the end of the periods of one, three, or 8 weeks' duration.

For the determination of the rats' ability to synthesize pyridine nucleotide, after they had been subjected to prolonged niacin-tryptophan deficiency, two groups of rats, which were fed the 0.093% tryptophan diet for a period of three months, were given, 16 hours and three hours prior to the time of killing, 100-mg doses of either nicotinamide, administered parenterally, or of nicotinic acid, administered orally.

Methods. Twenty-four-hour urine samples were analyzed for MNA by the method of Huff and Perlzweig ('47). At the end of the experiments the rats were decapitated. Three samples of each liver were immediately homogenized in ice cold trichloroacetic acid and aliquots of the homogenates were analyzed for pyridine nucleotide according to the method used by Robinson et al. ('47) which measures the concentration of DPN,[4] TPN[5] and MNA, expressed as DPN.

[3] Daily supplement in milligrams: thiamine·HCl, 0.10; riboflavin, 0.15; pyridoxine·HCl, 0.10; Ca pantothenate, 0.50; p-aminobenzoic acid, 0.40; inositol, 8.00; choline chloride, 30.0; biotin, 0.002; folic acid, 0.01; vitamin B_{12}, 0.0002; menadione, 0.05; α-tocopherol, 4.00; and cod liver oil, 250.

[4] Diphosphopyridine nucleotide.

[5] Triphosphopyridine nucleotide.

TABLE 1

Composition of experimental diets

Components	Diets				
	A	B[1]	C[1]	D[1]	E[2]
	%	%	%	%	%
Casein	12.0	8.2	6.0	1.2	1.2
Casein hydrolysate[3]	3.0	3.7	8.8	9.4	0.0
Gelatin	3.0	9.1	6.2	10.3	0.0
Glycine	2.6	0.0	0.0	0.0	0.0
Salt mixture[4]	2.5	2.5	2.5	2.5	2.5
Corn oil	8.0	8.0	8.0	8.0	8.0
Sucrose	added to bring to 100%				

[1] Amino acids were added to diets B, C and D in amounts necessary to bring the concentration of essential amino acids other than tryptophan approximately to that of diet A.

[2] DL-Methionine, 0.04%, was added.

[3] Acid, salt free. General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Hubell et al., ('37).

RESULTS

Experiment 1

Tryptophan requirement in pregnancy. The mean values of the weights and numbers of fetuses on the twentieth day of gestation, and for the weights and the pyridine nucleotide concentrations of the livers of pregnant and nonpregnant rats on the breeders' and the experimental diets are presented in table 2. In 100% of the rats of both the Wistar and the Osborne-Mendel strains fed the 0.136% tryptophan diet, the fetuses removed at autopsy on the twentieth day of gestation were alive and equal in number and weight to those of the corresponding control animals on the breeders' ration. In the Osborne-Mendel strain, there was no significant difference between the average maternal weight gains during pregnancy of the rats fed the two diets. In the Wistar strain, the average maternal weight gain was lower by 15% with the 0.136% tryptophan diet; the difference was significant at the 3% probability level. With the 0.093% tryptophan diet, on the twentieth day after mating only 50% of the animals were pregnant, and in these, on the average, 60% of the fetuses were alive and of normal size. Examinations of the uteri of the rats fed the 0.068% tryptophan diet revealed only pinpoint implantation sites, indicating that resorption had taken place early in the gestation period. These results point out that the minimal tryptophan intake required for pregnancy to proceed with normal growth and viability of the fetuses was more than 0.093% and possibly less than 0.136% for rats fed during gestation a niacin-free, 3% nitrogen diet, containing the amount of essential amino acids, other than tryptophan, which are supplied by 12% of casein, 3% of gelatin and 3% of casein hydrolysate.

Hepatic pyridine nucleotide concentration and urinary MNA excretion in pregnancy. The data presented in table 2 indicate that the pregnant rats of both strains, whether fed the breeders' ration or the niacin-free, low-tryptophan diets, did not differ in their liver pyridine nucleotide concentration from their nonpregnant controls. The average weight of the livers of the pregnant rats was 35% higher

TABLE 2

Average number and weight of fetuses, weight and pyridine nucleotide (PN) concentration of livers of pregnant and nonpregnant rats

No. of pairs of rats	Diet	Food intake		Fetuses		Liver weight		PN concentration	
		Pregnant	Non-pregnant	Weight	Number	Pregnant	Nonpregnant	Pregnant	Nonpregnant
		gm	*gm*	*gm*		*gm*	*gm*	*μg/gm*	*μg/gm*
					Wistar strain[1]				
15	Br.[2]	11	13	44 ± 4[3]	9.5 ± 0.6	9.3 ± 0.3	6.2 ± 0.1	673 ± 14	683 ± 16
16	A	11	13	47 ± 4	10.0 ± 0.7	8.0 ± 0.1	6.1 ± 0.0	669 ± 17	664 ± 12
					Osborne-Mendel strain[4]				
8	Br.[2]	13	16	40 ± 2	8.0 ± 0.4	12.8 ± 0.4	10.4 ± 0.5	797[5] ± 19	788[5] ± 20
6	A	13	16	46 ± 5	8.0 ± 0.4	12.5 ± 0.3	9.4 ± 0.3	781 ± 18	789 ± 19
8	B	13	15	12 ± 5	2.4 ± 0.9	10.9 ± 0.6	7.9 ± 0.2	749[5] ± 30	738[5] ± 29
6	C	12	12	0	0	9.0 ± 0.9	7.6 ± 0.5	671 ± 10	664 ± 28

[1] Average weight: nonpregnant, 220 gm; pregnant: fed diet Br., 280 gm; fed diet A, 271 gm; age, 4 to 6 months.
[2] Breeders' diet.
[3] Standard error.
[4] Average weight: nonpregnant, 303 gm; pregnant: fed diet Br., 371 gm; fed diet A, 371 gm; age, 5 to 8 months.
[5] Average of six.

than that of their nonpregnant controls. The differences were statistically significant ($P \leq 0.01$), even in the dietary group in which partial resorption occurred. Consequently, the total amount of hepatic pyridine nucleotide was higher by 35% in the pregnant animals. The weights of the livers expressed as a percentage of the rats' body weights averaged 3.13 and 2.79%, respectively, for the pregnant and nonpregnant animals of the Wistar strain, and 3.42 and 3.26%, respectively, for the Osborne-Mendel rats. Therefore the average values for the total amount of pyridine nucleotide per gram of body weight were higher by 11% in the pregnant than in the nonpregnant rats of the Wistar strain ($P \leq 0.03$), and were not significantly different in the rats of the Osborne-Mendel strain.

In the breeders' ration groups, the MNA output before pregnancy and at the end of the gestation periods averaged 114 ± 20 μg and 238 ± 67 μg, respectively, for the rats of the Wistar strain, and 350 ± 19 μg and 680 ± 38 μg, respectively, for the Osborne-Mendel strain. In the 0.136% tryptophan groups, the average pre-experimental MNA excretion of 150 ± 25 μg increased by the end of the 20 days' experimental period to 207 ± 39 μg in the pregnant animals, and decreased to 101 ± 28 μg in the nonpregnant rats of the Wistar strain. The respective values for the Osborne-Mendel rats were 340 ± 21, 451 ± 45 and 220 ± 20 μg. The increases in the output of MNA which resulted from pregnancy averaged 97%, and, consequently, were considerably larger than the corresponding augmentations in total hepatic pyridine nucleotide.

Experiment 2

Injection of ovarian hormones. Intramuscular administration of a combination of 4 mg of progesterone and 0.5 μg of estrone for 10 consecutive days to adult female rats, which were fed either the breeders' ration or the niacin-free, 0.136, 0.093, or 0.068% tryptophan diets, produced no detectable changes in the pyridine nucleotide concentration and the weight of the animals' livers. Since the results obtained in these experiments are in good agreement with those presented in table 2 for the control rats of the Osborne-Mendel strain, the data are not presented in a separate table. They indicate that the increases in urinary MNA excretion, which were found (Lojkin, '56) to result from the administration of ovarian hormones, are not paralleled by increases in hepatic pyridine nucleotide. Similarly to pregnancy, administration of ovarian hormones stimulates an increased output of MNA which is not accompanied by changes in the rat's liver pyridine nucleotide concentration.

Experiment 3

Effect of niacin-free, low-tryptophan diets. In table 3 are presented the changes in the concentrations of hepatic pyridine nucleotide, urinary excretion of MNA, sizes of livers and body weights of male rats, which resulted from the intake of niacin-free, low-tryptophan diets. The data obtained for the pyridine nucleotide concentration and the liver weight of each animal are expressed as the percentages of the corresponding values for its control on the breeders' ration, which was selected on the basis of matching MNA excretion levels and was killed and analyzed for hepatic pyridine nucleotide simultaneously. The levels of MNA excretion and the body weights of each animal at the end of the experimental periods are expressed as the percentages of its initial pre-experimental MNA and body weight values, respectively.

With the 0.136 and 0.093% tryptophan diets, marked decreases in the rats' MNA excretion occurred during the first and second weeks of the experiment. These were followed by fluctuations in the levels of MNA output, with values occasionally returning to as high as 80% of the original levels and then decreasing again. No significant changes in the hepatic pyridine nucleotide concentrations resulted from feeding diets A and B for periods of three weeks and one week, respectively. Feeding these diets for longer periods stimulated significant decreases in the rats' liver pyridine nucleotide concentrations. There was no noticeable lowering in the food intake and body weight of the animals fed these diets. The changes in weight paralleled those of their controls in the diet A group throughout the experiment, and in the diet B group, to the end of the period of three

TABLE 3

Average N[1]-methylnicotinamide (MNA) excretions and weights of male rats, expressed as percentages of the respective pre-experimental values,[1] and average pyridine nucleotide (PN) concentrations and weights of livers of experimental rats, expressed as percentages of the corresponding values[2] for the control rats

No. of pairs of rats	Diets	Duration of experiment	PN[3]	Weight livers[4]	$\frac{\text{MNA final}}{\text{MNA initial}} \times 100\%$	$\frac{\text{Weight final}}{\text{Weight initial}} \times 100\%$
		weeks	%	%	%	%
4	A	3	100±2[5]	98	62±9	114
5		8	87±1	90	68±7	127
4	B	1	98±2	98	50±4	102
5		3	91±2	97	58±11	103
5		8	83±1	94	33±9	107
5	C	3	80±1	89	11±0	98
4		8	74±2	68	4±0	94
5	D	3			9±0	82
5		8	55±0	62	3±0	72
5	E	3			17±2	83
5		8	68±1	64	27±8	72

[1] Average pre-experimental values: MNA, 396 μg/24 hours; weight of rats, 350 gm.
[2] Average values for the control rats: PN, 825 μg/gm; weight of livers, 12.9 gm.
[3] $\frac{\text{Liver PN of experimental rat}}{\text{Liver PN of control rat}} \times 100\%$.
[4] $\frac{\text{Weight liver of experimental rat}}{\text{Weight liver of control rat}} \times 100\%$.
[5] Standard error.

weeks. Further feeding of diet B resulted in smaller gain in weight in the experimental than in the control group.

Although the feeding of the niacin-free, low-tryptophan diet for a duration of several months decreased the rats' liver pyridine nucleotide concentration, it did not destroy the animals' ability to synthesize pyridine nucleotide. Parenteral administration of two 100-mg doses of nicotinamide to one group and oral administration of two 100-mg doses of nicotinic acid to another group of rats, which were fed the 0.093% tryptophan diet for three months, resulted, respectively, in approximately sixfold and threefold increases in the animals' hepatic pyridine nucleotide concentration over the values of the unsupplemented animals.

In the rats fed the 0.068% tryptophan diet, the hepatic pyridine nucleotide concentration decreased significantly by the end of the three weeks' experimental period. Feeding of diets C and D resulted in a sharp decrease in the animals' MNA output during the first week of the experiment, which was followed by further steady but more gradual lowering of the MNA values. With these two diets a decrease of 13% and of 20%, respectively, occurred in the animals' food intake. Similar partial inanition did not lower the levels of MNA excretion and hepatic pyridine nucleotide concentration in pair-fed controls.

The effect of the intake of niacin-free, low-tryptophan diets on the metabolism of nicotinic acid in the male rats in this experiment bears a resemblance to that observed in the female rats of both the Wistar and the Osborne-Mendel strain in experiment 1. In both the pregnant and nonpregnant animals, feeding of niacin-free, low-tryptophan diets for a period of 20 days produced no significant changes in the hepatic pyridine nucleotide concentrations at the 0.136% and 0.093% dietary tryptophan levels, but resulted in a definite reduction in the pyridine nucleotide concentration at the 0.068% tryptophan level (table 2). The MNA excretions, on the other hand, were found to decrease significantly following the intake of each of the diets, A, B and C for a period of 20 days.

Effect of amino acid imbalance. At the 0.014% level of tryptophan intake more drastic lowering of the hepatic pyridine nucleotide concentration and MNA excretion resulted from feeding diet D, which contained 18% of an imbalanced protein, than diet E, which contained 1% of a balanced protein (table 3). The pattern of the changes in the levels of MNA excretion in the diet E group was different from that of the diet D group and resembled the pattern observed in the rats on the 0.093% tryptophan diet, initial decreases in the excretion being followed by occasional increases in the MNA output. No significant difference was noted in the decrease in food consumption and in loss of weight between the rats in diet D and diet E groups.

DISCUSSION

Tryptophan requirements in pregnancy. Requirements for amino acids are not constant (Sauberlich and Salmon, '55), but increase as the levels of other essential amino acids and the total protein content of the diet are increased. Salmon ('54) found the tryptophan requirement of young rats to be 0.17% with a 20% protein diet and 0.13% with a 10.8% protein diet in the presence of niacin, and 0.30% and 0.19%, respectively, in the absence of niacin. According to Rao et al. ('59), the tryptophan requirement of weanling rats for maximum growth for a period of 21 days is 0.11% in the presence of niacin, on a 10% protein diet. Oesterling and Rose ('52) reported that 0.125% of tryptophan was inferior to 0.15% for the growth of weanling rats fed a niacin-free diet, which contained 16.4% of an amino acid mixture, and that no significant improvement in growth rate was noted when the level of tryptophan was increased from 0.15% to 0.20%. According to these investigators, addition of niacin stimulates growth at 0.10%, but not at 0.125% or higher levels of tryptophan intake. Since similarly to the growth of the weanling animals, pregnancy is a state of anabolic stress, the dietary requirements during gestation for the normal development of the fetuses might be compared with those of growth. The observations in this study of a minimal tryptophan requirement during gestation of more than 0.093% and possibly less than 0.136% for the normal growth of the fetuses are in fair agreement with the minimal requirements of 0.125 to 0.150% for the growth of weanling rats reported by Oesterling and Rose ('52). However, these values are lower than the 0.2% of tryptophan recommended by these investigators to insure maximal growth of the weanling rats. They are also lower than the intake of over 0.2% of tryptophan which, according to Pike ('51), is needed by the pregnant rat fed niacin-free, 14.7% casein hydrolysate diets for normal maternal weight gain and protection of the fetuses from congenital cataract.

Further work is being conducted in these laboratories to determine a more narrow range in the tryptophan requirement for pregnancy under the conditions of this experiment, as well as requirement at other levels of nitrogen and essential amino acids intake.

Hepatic pyridine nucleotide and MNA output in pregnancy. The observation that the increases in the total amount of hepatic pyridine nucleotide in the pregnant rat are considerably smaller than the augmentations in the MNA excretion suggest that the increases in the output of the metabolites of nicotinic acid in pregnancy cannot be attributed, at least entirely, to changes in the niacin reserves of the body. It appears more likely that the increases in the levels of excretion of niacin metabolites in pregnancy are related to the phenomenon of the general increases in the output of amino acids, which is known to occur in pregnancy. The augmentation in the output of MNA might serve as a means for the pregnant body of excreting an excess of tryptophan, after having converted it into niacin and its metabolites. The observed (Wertz et al., '58) greater efficiency in the conversion of tryptophan to niacin during pregnancy would facilitate this process of excretion.

Effect of niacin-free, low-tryptophan diets. Lowering of niacin-tryptophan intake decreased the MNA excretion before any significant changes occurred in the hepatic pyridine nucleotide concentration. Moreover the magnitude of the decreases in the MNA values was higher than that of the corresponding lowering of the pyridine nucleotide levels. Consequently, the uri-

nary output of MNA may be considered to be a more suitable measure of recent deficiency in niacin-tryptophan intake, whereas the pyridine nucleotide concentration could reflect only a more prolonged niacin-tryptophan deficiency. Results of a similar nature were obtained for human beings by Vivian et al. ('58). Their data indicate that the decreases in blood pyridine nucleotide resulting from the lowering of the niacin-tryptophan intake of the subjects are smaller and occur later than the corresponding decreases in MNA output.

Amino acid imbalance. In this investigation an amino acid imbalance resulting from the presence of tryptophan-free proteins in a niacin-free, 0.014% tryptophan diet stimulated decreases in the excretion of MNA and in the liver pyridine nucleotide concentration of rats. In experiments previously performed in these laboratories[6] an amino acid imbalance created by the addition of 10% of casein hydrolysate to a 15% casein diet produced no significant changes in the MNA excretion of pregnant and nonpregnant rats. Feeding of gelatin has been reported to have no effect on the levels of MNA excretion in the rat, (Schweigert and Pearson, '48; Rosen and Perlzweig, '49) and in man (Sarrett, '50). According to Pearson and Phornphiboul,[7] feeding of threonine resulted in lowering of the MNA output of rats. Morrison et al. ('60) noted that an amino acid imbalance created by the addition of gelatin or threonine to a 8% casein diet impaired the growth of young rats, but did not decrease their liver pyridine nucleotide concentration. Apparently the effectiveness of amino acid imbalances in lowering of MNA excretion and hepatic pyridine nucleotide concentration depends on the nature of the imbalance and on the levels of niacin-tryptophan intake, more pronounced decreases occurring at lower levels of intake.

SUMMARY

Rats were mated and either allowed to continue to consume a breeders' ration or fed from the first day of gestation niacin-free, 3% nitrogen diets, which differed in their content of tryptophan and were equal in their content of the other essential amino acids.

In 100% of the rats on a 0.136% tryptophan diet the young removed on the twentieth day of gestation were alive and did not differ in number and weight from those of rats fed the breeders' ration. With a 0.096% tryptophan diet pregnancy proceeded to the twentieth day in only 50% of the rats, and in these 60% of the fetuses were alive and of normal size. There was 100% resorption when diets containing 0.063% or less of tryptophan were fed. These observations suggested the conclusion that, under the conditions of these experiments, a tryptophan content of approximately 0.136% is required for pregnancy to proceed to term with normal growth of the fetuses.

No significant difference was noted between the concentrations of pyridine nucleotide in the livers of the pregnant rats and their nonpregnant controls in any of the dietary groups. The livers of the pregnant rats were heavier and, consequently, the total amount of hepatic pyridine nucleotide was larger in the pregnant than in the nonpregnant animals. The mean increase of the total content of hepatic pyridine nucleotide stimulated by pregnancy was 35%, whereas the corresponding increases in the urinary excretion of N^1-methylnicotinamide averaged 97%.

Intramuscular administration of a combination of 4 mg of progesterone and 0.5 μg of estrone for 10 consecutive days to female rats, which were fed either a breeders' ration, or niacin-free, low-tryptophan diets, induced no changes in the hepatic pyridine nucleotide concentration and in the size of livers of the rats in all the dietary groups.

Decreases in the levels of N^1-methylnicotinamide excretion which resulted from the feeding of niacin-free, low-tryptophan diets occurred sooner and were larger than the corresponding decreases in liver pyridine nucleotide concentration.

With a niacin-free, 0.014% tryptophan diet, the amino acid imbalance created by addition of tryptophan-free proteins produced a decrease in the levels of N^1-methylnicotinamide excretion and in the liver

[6] Unpublished data.

[7] Pearson, W. N., and B. Phornphiboul 1961 Niacin-tryptophan interrelationships in niacin deficiency induced by amino acid imbalance. Federation Proc., 20: 7 (abstract).

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Effect of Amino Alcohols and Methyl Donors on Liver Fat of Rats Fed Low-choline Diets Containing 2-Amino-2-methylpropanol[1,2]

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It has been found that 2-amino-2-methylpropanol (2A2MP) increases the severity of kidney hemorrhagic degeneration in rats deficient in choline (Wells, '55; Mulford and Outland, '57). The methyl donors, betaine, methionine and dimethyl-β-propiothetin (Wells, '56; Mulford, '55) were only slightly effective in overcoming the kidney damage due to high dietary levels of 2A2MP, whereas the methylated amino alcohols, choline, dimethylethanolamine, and monomethylethanolamine (Wells, '56; Outland et al., '59) were markedly effective. Wells ('56) reported that 2-amino-2-methylpropanol had a weak antilipotropic effect when 60 mg were injected intramuscularly per day for 14 days into 200-gm female rats receiving a choline-deficient diet. The kidneys of these animals appeared normal. Weanling rats, on the other hand, showed marked kidney hemorrhagic degeneration and no elevation in liver fat (Wells, '55).

We investigated the effect of feeding 2A2MP on liver fat of weanling male rats fed diets low in choline. The present communication shows that at the level of 1 mg of 2A2MP/gm of food the increase in liver fat was maximal. Higher levels of 2A2MP and lower levels resulted in a less than maximal increase in liver fat. We have also shown that the N-methyl derivatives of ethanolamine prevented the antilipotropic effect of a 1-mg level of 2A2MP and that the methyl donors and ethanolamine separately were quite ineffective. On the other hand, when betaine was incorporated into the diet containing added ethanolamine, it was able to overcome the antilipotropic action of 2A2MP effectively. Methionine plus ethanolamine was considerably less effective in overcoming the antilipotropic effects of the 2A2MP.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain born to females fed a complete stock ration[5] were used in this study. Animals 18 to 21 days of age and weighing 40 to 50 gm were placed in raised cages and fed experimental diets ad libitum for 7 days. Water was available to the animals at all times. Food consumption and body weights were recorded each day. At the end of the 7-day period the rats were decapitated and the kidneys examined for hemorrhage.

The livers were removed, weighed, ground with anhydrous sodium sulfate and extracted with chloroform in a Soxhlet extractor for 4 hours. Following extraction, the chloroform extract was filtered through fat-free filter paper into tared flasks, and the residue dried to constant weight. The dry residue weight represents the liver fat as recorded in tables 1, 2, and 3.

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[2] This investigation was supported by research grants from the National Institue of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service (A-219) and from the University of Kansas. A preliminary report of a part of this work has been presented: Longmore, W. J., and D. J. Mulford 1959 Fatty livers in rats receiving 2-amino-2-methyl-1-propanol. Federation Proc., *18*: 276 (abstract).

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TABLE 1

Effect of 2-amino-2-methylpropanol (2A2MP) on liver fat of young male rats consuming low-choline diet A

2A2MP/ gm of food	No. of rats	Rats with hemorrhagic kidneys	Avg wt of liver fat	Liver fat as % of fresh liver[1]	Avg daily wt increase	Avg daily food intake
mg		%	*mg*		*gm*	*gm*
0	348	4	436	11.1 ± 0.36	3.8	6.4
1	668	26	826	16.3 ± 0.19	3.5	6.2
2	108	58	546	11.0 ± 0.41	3.1	5.6
3	36	75	365	8.0 ± 0.34	2.4	5.1

[1] Mean ± standard error.

TABLE 2

Effect of 2-amino-2-methylpropanol (2A2MP) on liver fat of young male rats consuming diets A and B containing either an added amino alcohol or methyl donor

Diet	Supplement/ gm of food	2A2MP/ gm of food	No. of rats	Avg wt of liver fat	Liver fat as % of fresh liver[1]	Avg daily wt increase	Avg daily food intake
	$\times 10^{-3}$ *mmole*	*mg*		*mg*		*gm*	*gm*
A	7 E[2]	0	108 (11)[3]	443	11.0 ± 0.55	3.7	6.2
		1	168 (26)	720	14.8 ± 0.45	3.5	6.2
A	14 E	0	84 (0)	328	8.1 ± 0.34	4.1	6.5
		1	60 (5)	732	15.1 ± 0.33	4.0	6.6
A	21 E	0	36 (0)	273	6.8 ± 0.39	3.8	6.0
		1	12 (9)	636	13.4 ± 1.10	3.8	6.3
A	7 MME[2]	0	60 (0)	198	5.5 ± 0.55	3.4	5.8
		1	108 (0)	298	7.2 ± 0.56	3.7	6.0
A	7 DME[2]	0	72 (0)	142	3.8 ± 0.41	3.7	6.0
		1	60 (0)	190	4.7 ± 0.41	3.8	6.0
A	7 Chol.[2]	0	84 (0)	95	2.6 ± 0.17	3.5	5.8
		1	120 (0)	162	3.9 ± 0.18	3.5	5.8
B	0	0	72 (0)	156	4.5 ± 0.33	3.4	5.9
		1	180 (21)	570	12.1 ± 0.35	3.4	5.8
A	52 Meth.[2]	0	36 (0)	196	5.8 ± 0.43	3.3	5.9
		1	60 (0)	629	13.6 ± 0.86	3.2	6.1
B	52 Meth.	1	12 (17)	633	12.5 ± 2.10	2.9	5.1
A	52 Bet.[2]	0	60 (0)	182	4.8 ± 0.43	3.8	6.5
		1	144 (0)	543	11.1 ± 0.33	4.0	6.7

[1] Mean ± standard error.
[2] E = ethanolamine, MME = monomethylethanolamine, DME = dimethylethanolamine, Chol. = choline chloride, Meth. = L-methionine and Bet. = betaine hydrochloride.
[3] Number in parentheses represents the percentage of animals having kidney lesions.

The basal low-choline diet (A) was similar to that of Mulford and Griffith ('42) and consisted of dry brewer's yeast,[6] 6; agar, 2; salt mixture,[7] 4; lard, 19.9; fortified fish liver oil,[8] 0.1; calcium carbonate, 1.0; L-cystine, 0.3; casein,[9] 18; and sucrose 48.7%. A second diet (B) used in some of our experiments was the same as diet A except that it contained 42% of casein and 24.7% of sucrose. Supplements of methyl donors and amino alcohols as well as 2A2MP were dissolved in water and mixed into diets with the lard.

RESULTS

Effect of the addition of various levels of 2-amino-2-methylpropanol on the liver fat of young male rats consuming diet A. It was of interest to us that Wells ('55) was unable to observe fatty livers in young rats consuming a low-choline diet containing 1% of 2A2MP. This led to a study in which levels of 1, 2, and 3 mg of

[6] Anheuser-Busch, Inc., strain G.
[7] Salt Mixture XIV, General Biochemicals, Inc., Chagrin Falls, Ohio.
[8] Natola, Parke-Davis and Company, Detroit.
[9] Vitamin Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 3

Effect of methionine and betaine on liver fat of young male rats consuming diets A and B containing added ethanolamine and 1 mg of 2-amino-2-methylpropanol per gm of food

Diet	Supplement/ gm of food	Ethanol-amino added/ gm of food	No. of rats	Avg wt of liver fat	Liver fat as % of fresh liver[1]	Avg daily wt increase	Avg daily food intake
	$\times 10^{-3}$ mmole	$\times 10^{-3}$ mmole		mg		gm	gm
A	26 Meth.[2]	7	12	625	13.0 ± 0.16	4.0	7.2
A	26 Bet.[2]	8	12	431	8.9 ± 1.91	4.1	6.0
B	0	7	60	395	10.4 ± 0.70	3.6	5.7
A	52 Meth.	7	12	578	11.7 ± 1.65	3.4	5.4
A	52 Bet.	7	36	271	5.8 ± 0.49	4.0	6.2
A	52 Bet.	8	12	146	3.8 ± 0.36	4.0	7.0
B	0	14	12	540	11.1 ± 0.48	4.0	6.4
A	52 Meth.	14	12	479	10.4 ± 0.32	3.9	6.5
A	52 Bet.	14	24	187	4.4 ± 0.26	3.9	6.4
A	7 Bet.	12	12	653	13.3 ± 0.98	4.2	6.4
A	26 Bet.	12	12	332	7.5 ± 0.73	4.0	6.3
A	52 Bet.	12	12	129	3.4 ± 0.10	4.0	6.8
A	52 Bet.	21	12	146	3.4 ± 0.09	3.9	6.2
B	52 Meth.	7	12	381	9.0 ± 1.75	3.5	5.5

[1] Mean ± standard error.
[2] Meth. = L-methionine, Bet. = betaine hydrochloride.

2A2MP/gm of food were added to diet A and fed to young male rats for 7 days. Table 1 shows that the addition of 1 mg of 2A2MP/gm to diet A increased the liver fat from 11.1 to 16.3% and increased the incidence of kidney lesions from 4 to 26%. As the level of 2A2MP was increased to 2 and 3 mg the incidence of kidney lesions increased, but the liver fat decreased from the high level (16.3%) observed when 1 mg of 2A2MP/gm was added to the diet. Since both the rate of increase in body weight and the intake of food decreased with increase of 2A2MP, it might be presumed that when the animals became sick, they refused to eat, lost weight and therefore deposited less fat in the liver. It might also be postulated that 2A2MP itself will lower the liver fat when fed at high levels, but evidence for this is not at hand. The results shown in table 1 perhaps will shed some light on the results of Wells who fed 2A2MP at 10 mg/gm of food and observed no elevation of liver fat even though the incidence of kidney lesions was extremely high.

Effect of the addition of 1 mg of 2-amino-2-methylpropanol per gm of food on liver fat of young male rats consuming diets containing either an added amino alcohol or methyl donor. Experiments were carried out in which 1 mg of 2A2MP/gm of food was added to diet A containing either an added amino alcohol or an added methyl donor. The level of 1 mg of 2A2MP/gm was chosen because it had led to the greatest elevation of liver fat (table 1). The effects of 2A2MP on liver fat of rats consuming these diets for 7 days are shown in table 2. In this table, it is shown that in the absence of 2A2MP both the N-methyl derivatives of ethanolamine and the methyl donors at 7×10^{-3} mmole and 52×10^{-3} mmole/gm of food, respectively, maintained the liver fat at a low level. Ethanolamine at 7×10^{-3} mmole had little effect and at higher levels had only a moderate effect. When 1 mg of 2A2MP was added to the diets containing these substances, the liver fat was increased in all animals. The least change occurred in the liver fat of animals receiving the added N-methyl derivatives of ethanolamine and the greatest change occurred in the liver fat of animals receiving either the added methyl donors or added ethanolamine. In an experiment not shown in table 2. 1 mg of 2A2MP/gm was added to diet A containing 52×10^{-3} mmole of dimethyl-β-propiothetin hydrochloride. The liver fat of the animals increased from 4.6 to 10.9%.

Of the amino alcohols choline was slightly more effective in maintaining the

liver fat at a low level than dimethylethanolamine which in turn was more effective than monomethylethanolamine. When a comparison is made between the results obtained with methionine and those obtained with betaine, one sees evidence that the lipotropic activity of methionine was perhaps more inhibited by 2A2MP than betaine. Some of the rats consuming ethanolamine had hemorrhagic kidneys whereas those consuming its N-methyl derivatives had none. Very few of the animals consuming added methyl donors had hemorrhagic kidneys.

The liver fat of animals consuming diet B (table 2) containing no 2A2MP was approximately 4.5%. None of the animals had kidney lesions. When 1 mg of 2A2MP was added/gm of diet B, the liver fat was increased to approximately 12.1% and the incidence of kidney lesions was increased to 21%. Similar results were obtained when 2A2MP was added to diet B to which 52×10^{-3} mmole of L-methionine had been added. The fact that diet B without added 2A2MP maintained the liver fat at close to the normal level, but failed to do so when 2A2MP was added suggests that the methionine level in the 42% casein of diet B was effective in reducing liver fat in the absence of 2A2MP but was ineffective in the presence of 2A2MP.

The results of these experiments indicate that 2A2MP increased the requirement for the naturally occurring amino alcohols and that the N-methyl derivatives of ethanolamine met the increased requirement more easily than ethanolamine. They also indicate that 2A2MP reduced the lipotropic activity of methyl donors. It is postulated that the reduced lipotropic effect of the methyl donors by 2A2MP was due to the decreased availability of amino alcohol. When less natural amino alcohol was available, the methyl donors were unable to donate their methyl groups to the amino alcohol acceptor and as a result were less active in reducing liver fat.

Effect of methionine and betaine on liver fat of young male rats consuming diets containing added ethanolamine and 1 mg 2A2MP per gm of food. Because it was found that 2A2MP inhibited the lipotropic activity of the methyl donors, betaine, methionine, and dimethyl-β-propiothetin and increased the requirement for the amino alcohols, ethanolamine, monomethylethanolamine dimethylethanolamine, and choline, we decided to study the effect of methyl donors on liver fat of animals receiving diets containing both 1 mg of 2A2MP/gm and various levels of the amino alcohols. Since the N-methyl derivatives of ethanolamine by themselves were highly effective in overcoming the antilipotropic action of 2A2MP, ethanolamine which was much less effective was chosen as the amino alcohol for study. Table 3 shows the effects of adding either betaine hydrochloride or L-methionine to diets containing both 1 mg of 2A2MP and ethanolamine added at levels ranging from 7×10^{-3} mmole to 21×10^{-3} mmole/gm of food.

When the results of groups receiving betaine are compared with those receiving the same levels of methionine, betaine was much more effective than methionine in overcoming the antilipotropic action of 2A2MP in the maintenance of liver fat at near normal levels. Diet B containing 42% of casein and thus approximately 52×10^{-3} mmole more of methionine/gm than diet A behaved essentially the same as diet A to which 52×10^{-3} mmole of methionine had been added per gram. The animals receiving diet B to which 52×10^{-3} mmole of methionine/gm of food had been added were also high in liver fat at approximately 9.0%. The results recorded in tables 2 and 3 suggest that although the lipotropic activity of betaine was inhibited by 2A2MP, it was not inhibited when ethanolamine was present in adequate amounts. In all experiments the observed reduction in liver fat when both betaine and ethanolamine were together in the diet was always greater than the calculated reduction based on the combined effects of both when fed separately. This would indicate the ethanolamine enhanced the activity of betaine and vice versa. Methionine appeared to be much less effective than betaine when fed both in the presence and in the absence of added ethanolamine.

DISCUSSION

The literature pertaining to choline deficiency and the effects of the amino alco-

hols, monomethylethanolamine, dimethylethanolamine, and choline and of the methyl donors, betaine, methionine and the thetins in overcoming its symptoms is quite extensive and has been reviewed (Griffith and Nyc, '54; Griffith, '58; Harper, '58). Ethanolamine at high levels has been found to increase the severity of kidney damage and at the same time to decrease liver fat, whereas at low levels it was relatively inactive (Mulford et al., '59). In the present study 1 mg of 2A2MP/gm of food not only increased the incidence of kidney lesions but also elevated the liver fat. The N-methyl derivatives of ethanolamine were markedly active in overcoming these changes, whereas the methyl donors and ethanolamine were relatively inactive. Choline was slightly more effective than dimethylethanolamine, which in turn was somewhat more effective than monomethylethanolamine. When betaine was added to the diet containing added ethanolamine, inhibition by 2A2MP was prevented. The same was not true when methionine was added instead of betaine.

One might interpret these results as indicating that 2A2MP increases the need of amino alcohols perhaps for the synthesis of natural liver phospholipid. A great deal of circumstantial evidence suggest that the lipotropic activity of choline depends upon its incorporation into phospholipid (Griffith and Nyc, '54). Of interest is the work of Longmore and Mulford ('60, '62) who reported that 2A2MP was not only incorporated into liver phospholipid but it also inhibited the incorporation of ethanolamine-1, 2-C^{14} and dimethylethanolamine-1, 2-C^{14} and to a slight extent choline-1, 2-C^{14} into liver phospholipid.

The work of Wilson et al. ('60), Gibson et al. ('61), Bremer and Greenberg,[10] Bremer et al. ('60) and Artom and Lofland ('60) suggests that ethanolamine and its N-methyl derivatives, monomethylethanolamine and dimethylethanolamine are incorporated into phospholipids before being methylated. In light of this work the inhibition of the methyl donors by 2A2MP might be explained on the decreased availability of the phospholipids of these specific amino alcohols for methylation. When adequate ethanolamine was added to the diet containing 2A2MP and added betaine, the marked reduction in liver fat suggests that phosphatidyl ethanolamine was formed and betaine in turn methylated it. That methionine was not able to do the same thing suggests that methionine follows a different pathway in methylation than betaine. Of interest is the recent report of Wells and Remy ('61) that the chronic administration of 2-amino-2-methylpropanol to young rats produced an inhibition of the incorporation of the methyl group of methionine into lipid-bound choline of liver, kidney and other tissues. The methyl group of betaine on the other hand was not inhibited. The work of Harper and Benton ('56) and of Young et al. ('56) which showed that betaine was more effective than methionine in preventing fatty livers due to choline deficiency supports this suggestion. Stekol ('58) found dimethylethanolamine as the direct *in vivo* acceptor of the methyl of methionine in rats on diets deficient in folic acid and receiving methionine-CH_3-C^{14}. Ethanolamine and monomethylethanolamine were without effect.

SUMMARY

When 2-amino-2-methylpropanol (2A2MP) was added to diets low in choline at a level of 1 mg/gm of food both the incidence of kidney lesions and the amount of liver fat were elevated. At higher levels of 2A2MP the kidney lesions were even more severe but the liver fat was lower than that due to 1mg 2A2MP/gm of food. The addition of N-methyl derivatives of ethanolamine, namely, monomethylethanolamine, dimethylethanolamine and choline was markedly effective in preventing the elevation of liver fat due to 1 mg of 2A2MP/gm of food. Ethanolamine had only a slight effect. The methyl donors, betaine, methionine and dimethyl-β-propiothetin when added to the diet containing 1 mg of 2A2MP/gm of food were also only slightly effective. When added to diets containing 1 mg of 2A2MP/gm and adequate ethanolamine, betaine reduced the liver fat to a great extent, whereas methionine reduced it only slightly. The re-

[10] Bremer, J., and D. M. Greenberg 1960 Mechanism of choline biosynthesis. Federation Proc., *19:* 232 (abstract).

sults suggest that dietary 2A2MP increased the requirement for ethanolamine and for it N-methyl derivatives, choline, dimethylethanolamine and monomethylethanolamine.

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Effect of Moldy Diet and Moldy Soybean Meal on the Growth of Chicks and Poults

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Grain that has undergone spontaneous heating due to the growth of various fungi is considered unsuitable for human foods, but large quantities are used widely in livestock and poultry feeds. Some animals consuming certain lots of moldy grain have developed characteristic symptoms and died (Ewing, '51; Semeniuk, '54; Morrison, '56). These conditions were attributed to a toxicosis. Other animals consuming moldy grains were affected only slightly or not at all. The effect of moldy grain on the growth of different species of animals has been variable. An excellent review (Forgacs, '62) on the mycotoxicoses that have developed in different species of animals has been published recently.

Renewed interest has developed in the possible toxicity of moldy feeds because of the widespread mortality in poults that received certain ground nut meals. This disease has been called "Turkey X" disease (Blount, '61) and the toxic substance has been associated with the growth of *Aspergillus flavus* (Lancaster et al., '61).

In view of the high mortality in poults receiving certain ground nut meals and the retarded growth obtained in this laboratory in chicks receiving a moldy diet, it was decided to investigate the nutritive properties of moldy soybean meal using poults as the test animal. A summary of the tests with chicks and the results with poults receiving diets containing moldy soybean meal are described in this report.

EXPERIMENTAL

Preparation of moldy feed and soybean meal. A moldy diet was used in the tests with chicks and moldy soybean meal was used in those with poults. The procedure used to mold the soybean meal was briefly as follows: Two hundred pounds of soybean meal were mixed and then divided into two lots. One lot containing 10% moisture was stored in covered containers at ambient temperature as a control for the moldy meal. There was no fungal growth in this lot and it is designated as the control. The second lot, after adjusting the moisture content to 19% by the direct addition of water, was placed in a series of 4-liter Dewar flasks. The flasks containing the meal were then stored in a heating cabinet at 31°C and 78% relative humidity for 6 weeks. Growth of the naturally occuring fungi produced heating in 5 to 6 days and the temperature of the meal in the flask was an average of 43°C during the entire storage period. This continuous high temperature shows that fungi were growing during the entire storage period. To insure that all the soybean meal within a flask was subjected to fungal activity, the contents of each flask were mixed thoroughly at the end of three weeks. At the end of 6 weeks, the meal was dried in an air circulating drier at 40°C for 24 hours. The entire lot which had a moisture content of approximately 10% was reground, mixed thoroughly and stored at room temperature until used. This same procedure was used to mold the diet for chicks with the exception that the feed was allowed to mold for periods of 2, 4, 6 and 10 weeks.

Using Czapek solution agar and potato dextrose agar, the predominant fungi on moldy soybean meal were *Penicillium* sp. and *Aspergillus glaucus* group.

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Extracted soybean meal. If the retarded growth was due to a toxic substance, it appeared that it might be possible to remove it by extraction with some solvent. With this possibility in view, moldy and control soybean meals were each extracted with 70% ethanol. In this extraction, 5 kg of soybean meal were stirred one hour with 10 liters of 70% ethanol. After the mixture had settled for an hour, the extract was filtered off on a large Buchner funnel. This procedure was repeated 4 times and the combined extracts were concentrated to 33% moisture. The concentrate from the control soybean meal is designated as control extract and that from the moldy meal is designated as moldy extract. The residues left from the extraction were dried to approximately 10% moisture at a maximum temperature of 45°C. The residue from the control meal is designated control residue and that from the moldy meal is designated moldy residue.

Care of poults. Day-old white broad breasted mixed sex (Nicholas) poults obtained from a commercial hatchery were used to test the effect of the moldy soybean meals and moldy residues. Ten poults per group with essentially the same average weight were housed in electrically heated battery-type brooders. Feed and water were supplied ad libitum and the poults were weighed weekly during the experimental period indicated in the tables. In these studies, deaths occuring during the first week were due usually to a weak poult or an accident and these poults were not counted in the observation. The composition of a typical diet is given in table 1. Control and moldy soybean meal and the residues were substituted for the soybean meal in this diet at a level of 35%.

TABLE 1

Composition of typical diet

	gm
Sorghum grain (milo)	58.0
Soybean meal[2]	35.0
Steamed bone meal	2.0
NaCl	0.5
$MnSO_4H_2O$	0.1
Corn oil[3]	0.5
DL-Methionine	0.2
Choline chloride	0.3
Vitamins[1]/100 gm	
Vitamin A	3000IU
Vitamin D_3	400ICU
	mg
Menadione	0.75
α-Tocopherol	2.0
Thiamine·HCl	1.0
Riboflavin	1.0
Pyridoxine·HCl	1.0
Ca pantothenate	4.0
Niacin	2.0
Biotin	0.02
Folic acid	0.2
Vitamin B_{12}	0.01
Inositol	10.0

[1] The author is indebted to Merck and Company, Rahway, N. J., for generous supplies of vitamins; to Lederle Laboratories, Division of American Cyanamid, Pearl River, N. Y. for folic acid.
[2] Control and moldy soybean meal analyzed 48% and 57% protein, respectively.
[3] Mazola, Corn Products Company, New York.

RESULTS AND DISCUSSION

Moldy diet for chicks. A few tests have been carried out in this laboratory on the effect of a moldy diet on the growth of chicks. The details of these tests were described in another paper (Richardson and Webb, 62) and only a brief summary of the results will be given in this report. In these tests, a diet that was adequate for the growth of chicks was stored under moisture and temperature conditions which permitted the growth of the naturally occurring fungi. Storage periods of 2, 4, 6 and 10 weeks were used. As a control for the moldy diet, a second portion of the same diet was stored under the same temperature conditions, but the moisture content was below that which permitted the growth of fungi.

The diet, which had been allowed to mold two weeks, supported a rate of growth essentially equal to that of the control or nonmoldy diet. When the diet was allowed to mold 4, 6, or 10 weeks, the chicks grew at a progressively slower rate as the molding period increased. Supplementing the moldy diet with known vitamins, protein or fat did not improve it to a significant degree. There were no deficiency symptoms in the chicks or evidence of a disease to account for the retarded growth and it was tentatively concluded that the fungi produced some substance which was sufficiently toxic to retard the growth of chicks.

Moldy soybean meal for poults. The average gain in weight at three weeks of poults receiving various soybean meals and residues is summarized in table 2. The data for groups 1 and 2 show that the nutritive value of the meal was decreased markedly by the molding process. Poults receiving either control (group 3) or moldy residue (group 4) grew at a slower rate than those receiving control soybean meal (group 1). Five of 10 poults receiving the moldy residue died during the second and third week. None receiving the control residue died.

Addition of the moldy extract to the moldy residue (group 5) did not improve the rate of growth, but addition of the moldy extract (group 7) to control soybean meal increased the rate of growth slightly over that obtained by the control soybean meal (group 1). Addition of control extract to control soybean meal (group 6) supported essentially the same rate of growth as the control meal (group 1). These data show that extraction of the moldy soybean meal with 70% ethanol did not remove any substance which retarded the growth of poults when the extract was fed with control soybean meal. On the other hand, the high mortality in groups 4 and 5 which received the moldy residue suggests that the residue may have been more detrimental to the poults than the original moldy soybean meal (group 2).

Pathology. Two of the poults receiving the moldy residue (table 2, group 4) appeared moribund and were killed for gross and histopathological examination. The following changes were observed in one poult. There was focal myocardial necrosis with the formation of small round homogeneous pale-pink globules of proteinaceous-type residues. In the kidney there was extensive degeneration or necrosis of tubule epithelium and some degeneration in the glomerular tufts. The viable epithelium of tubules was very swollen. In the liver lipidosis was observed that was more severe in some areas than in other areas. Nucleoli of the hepatic cells were very distinct. The final diagnosis was a toxic hepatitis and nephritis. The symptoms in the other poult were similar but less severe. Histological examination of the tissue of the poults in this group (no. 4, table 2) which survived to three weeks showed no characteristic changes from poults receiving control soybean meal. Histopathological examinations were carried out on the livers of 28 additional poults receiving control soybean meal and 28 receiving moldy meal. The hepatic epithelium in most of the poults from both groups exhibited morphological alterations. The cytoplasm of the affected epithelial cells was distended by an excessive amount of water soluble substance (presumably glycogen). The chromatin of the nuclei was marginated and the nucleoli were enlarged. These lesions were most severe in two of the last 5 poults killed at 19 days. Livers of 10 poults receiving moldy soybean meal and 0.8% of lysine were less severely affected although increased fat or glycogen content was demonstrable in some hepatic epithelial cells.

Amino acid supplements. Total amino acids were determined in acid hydrolyzed control and moldy soybean meals by the ion exchange procedure (Moore et al.,

TABLE 2

Various fractions of control and moldy soybean meals (SBM) in diets for poults

Group no.	Fractions of soybean meal	No. of poults		Avg gain, 3 weeks
		Initial[1]	Final	
				gm
1	Control SBM	20	20	240
2	Moldy SBM	20	19	73
3	Control residue	10	10	131
4	Moldy residue	10	5	118
5	Moldy residue + moldy extract[2]	8	6	98
6	Control SBM + control extract[2]	9	9	238
7	Control SBM + moldy extract[2]	19	19	292

[1] Initial no. = number surviving at one week.
[2] Fed at equivalent of three times 35% soybean meal.

'58). The meals were hydrolyzed by refluxing 1.5 gm of soybean meal with 150 ml of constant boiling hydrochloric acid for 24 hours. Hydrochloric acid was removed by evaporating under reduced pressure. Distilled water was added and the evaporation was repeated 6 times. Free amino acids were obtained from the meals with 70% alcohol by the method described by De Vay ('52). The amino acids were determined in the extract by the ion exchange procedure. In general, most of the free amino acids were slightly higher in moldy meal than in the control meal, but the relative amounts of free amino acids in both meals was small in comparison with the total amino acids. The concentration of total amino acids in both meals is given in table 3.

Of the essential amino acids, lysine and probably arginine were the only ones that appeared to be significantly less in the moldy meal. Using the data for lysine in table 3, the amount of lysine supplied by the control and moldy soybean meals was 0.830% and 0.637%, respectively. The estimated amount of lysine supplied by the sorghum grain was approximately 0.174% in each case. The lysine requirement for poults has been shown to range from approximately 1.1 to 1.46% (Almquist, '52; Griminger and Scott, '59).

TABLE 3

Amino acid content of control and moldy soybean meals

Amino acid	Control	Moldy
	mg/gm	*mg/gm*
Aspartic acid	53.6	47.2
Threonine	17.9	16.9
Serine	31.1	23.1
Proline	15.4	14.9
Glutamic acid	74.4	70.9
Glycine	20.3	20.6
Alanine	20.6	19.7
Valine	17.3	19.0
Cystine	—	—
Methionine	6.0	6.7
Isoleucine	21.1	21.6
Leucine	35.0	32.7
Tyrosine	15.4	14.1
Phenylalanine	23.4	25.9
Lysine	23.7	18.2
Histidine	8.8	8.5
Arginine	38.3	24.8

These data suggested that lysine was present in borderline amount and a preliminary test was run supplementing the diet containing the moldy meal with 0.8% of lysine. With this amount of lysine added, the moldy meal supported a rate of growth equal to that obtained with the control meal.

One group of 6 broad breasted bronze poults was given the moldy soybean meal diet for 4 weeks. A photograph of a poult from this group is given in figure 1 and the development of white bands in wing feathers shows that the diet supplied inadequate lysine.

After the preliminary test had shown that lysine essentially corrected the inadequacy of the moldy soybean meal, another series was set up to test the effect of lysine and arginine on both control and moldy meals. There were 10 poults per group and the experimental period was 4 weeks. These results are summarized in table 4. As in previous tests, growth of poults (series 1) receiving moldy soybean meal was severely retarded. Supplementing with 0.6% of lysine (series 2) improved the moldy meal but did not correct it completely. When lysine was increased to 0.8% (series 3), the poults receiving the moldy meal grew slightly faster than those receiving the control meal. When the lysine was further increased to 1.2%, growth of poults receiving the moldy meal was depressed. Since this amount did not depress growth of the poults receiving control soybean meal, it seemed probable that some amino acid other than lysine may have been affected by growth of the fungi. Total amino acid analysis showed that moldy meal was lower in arginine than the control meal and tests were run to determine whether this amino acid might be limiting under some conditions. Both moldy and control soybean meals were supplemented with 1.0% (series 5) and 1.5% (series 6) of arginine. Growth of poults receiving the moldy meal was definitely increased with 1.5% of arginine, but the most rapid rate of growth (series 7) was obtained when the moldy meal was supplemented with 0.8% of lysine and 1.0% of arginine. These data show that retarded growth of

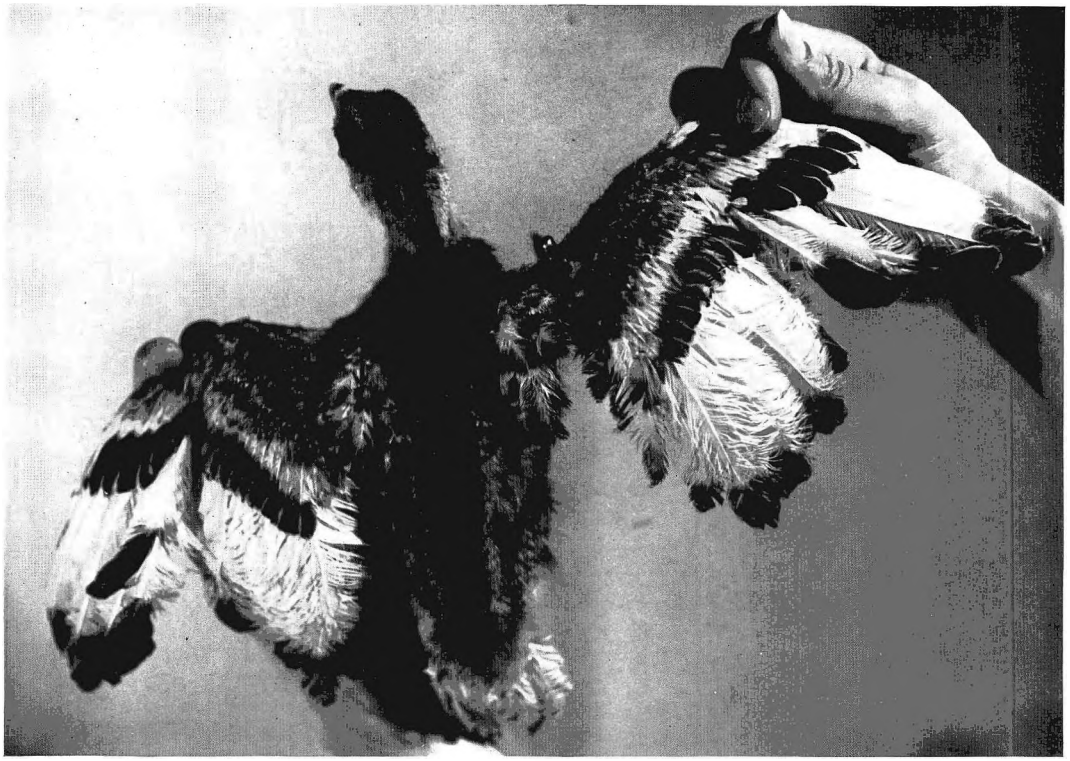

Fig. 1 Broad breasted bronze poult that had received a moldy soybean meal diet for 4 weeks.

TABLE 4

Lysine and arginine as supplements to control and moldy soybean meals in diets for poults

Amino[1] acids	Series no.	Control		Moldy	
		No. of poults[2]	Gain[3]	No. of poults[2]	Gain[3]
%			*gm*		*gm*
None	1	10	510	9	223
Lysine, 0.6	2	8	535	8	411
Lysine, 0.8	3	9	501	10	518
Lysine, 1.2	4	9	501	10	461
Arginine, 1.0	5	8	519	7	238
Arginine, 1.5	6	8	515	8	399
Lysine, 0.8 + Arginine, 1.0	7	5	501	9	543

[1] L-Lysine and L-arginine monochlorides were fed, but the amounts shown represented the calculated free base.
[2] There were no deaths in any groups after the first week.
[3] Observed average gain at 4 weeks.

poults receiving moldy soybean meal is due primarily to some important change in the availability of lysine. Arginine may be involved also, but this amino acid does not necessarily become a limiting factor for the growth of poults until a fairly large excess of lysine is added. Further studies are needed to determine how the growth of fungi affect the amino acids so that they become limiting factors for the growth of animals. The amino acid analysis indicated that the effect is more than merely destruction of the amino acids. The possibility that some fungi may produce a

metabolite that is antagonistic to lysine has been considered, but the observations with 70% ethanol-extracted meal appears to discredit this probability.

The fact that maximal growth was not obtained with less than 0.8% of lysine indicates that the moldy meal contained very little available lysine. Whether the problem with moldy soybean meal is related to "Turkey X" disease produced by certain peanut meals has not been established.

SUMMARY

Retarded growth occurred in poults that received a diet containing moldy soybean meal. This moldy meal was produced by subjecting commerical soybean meal to fungal growth for 6 weeks. In general, mortality was low, but gross and histopathological examination of two poults that were moribund showed a toxic hepatitis and nephritis. Poults receiving the moldy soybean meal and 0.8% of lysine grew at a normal rate and the livers were less severely affected. Supplementing the moldy meal with 1.5% of arginine indicated that the availability of this amino acid also may have been affected by the fungal growth. When 1.2% of lysine was added to the moldy meal, arginine definitely became a limiting amino acid for growth.

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Biochemical and Autoradiographic Studies on DNA Metabolism in Vitamin E-deficient Hamsters[1]

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Recent studies on the incorporation of P^{32} orthophosphate, formate-C^{14} and glycine-1-C^{14} (Dinning et al., '55, '56, '58) indicate that the metabolism of deoxyribonucleic acid (DNA) in bone marrow and muscle is altered in vitamin E-deficient animals. The interpretation of the results of these investigations is complicated by two considerations, namely, (a) differences in the incorporation of formate or glycine into DNA may result not only from alterations in the biosynthesis of DNA but also from alterations in the biosynthesis of precursors of DNA (e.g., purines), and (b) the cellular populations of normal and deficient muscle are not comparable since the skeletal muscle of vitamin E-deficient animals is characterized by the presence of degenerative lesions infiltrated by various types of inflammatory cells. It is difficult to decide to what extent differences in the metabolism of DNA in deficient muscle are due to changes in DNA turnover in the cells normally constituting muscle or reflect alterations in the cell population, or both.

The use of H^3-thymidine in the study of DNA metabolism surmounts the above-mentioned difficulties in interpretation. The cell type actively synthesizing DNA can be identified since H^3-thymidine is incorporated exclusively and directly into DNA (Reichard et al., '51) and, as a weak beta-emitter, is especially suitable for autoradiographic studies. In the present investigation, H^3-thymidine was injected into hamsters fed a diet deficient in vitamin E or into hamsters fed a deficient diet supplemented with vitamin E. At various time intervals, animals from each group were killed and autoradiographs prepared from selected tissues. In addition, DNA was isolated from various organs and tissues and its specific radioactivity determined. Thus, it was possible to correlate changes in the specific activity of DNA isolated from a given organ with the pattern of labeling of different cells of that organ.

METHODS

Thirteen Syrian golden hamsters were maintained after weaning with a diet deficient in vitamin E for 120 days. The percentage composition of the diet was as follows: casein, 20; cornstarch, 30; rolled oats, 20; brewer's yeast, 7.5; salts, 2.5; stripped lard, 14; cod liver oil, 6. Another group of 7 hamsters from the same litter was fed the same diet supplemented daily with 1 mg of α-tocopherol. Two days before the injection of the isotope, each of 6 vitamin E-deficient hamsters was given 25 mg of α-tocopherol orally on each of two successive days. These hamsters will henceforth be called previously deficient, vitamin E-treated hamsters.

Each hamster received a single intraperitoneal injection of H^3-thymidine, 100 μc/100 gm of body weight[3] (specific activity 3 μc/mmole). The animals weighed 146 ± 18 gm and were housed in metabolism cages after the injection. Urine was then collected over 24-hour intervals. One male and one female from each group was killed at one, three and seven days after injection of H^3-thymidine. One defi-

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[3] Purchased from Schwarz Bioresearch Corporation.

cient female and one normal male hamster was killed 30 days after the injection.

The DNA was isolated from liver, intestine, bone marrow and testis and its specific activity determined as described previously (Gerber et al., '60a). Since in the case of muscle the yields of DNA obtained by this method were unsatisfactory, DNA was isolated according to the procedure of Schneider ('45).

Smears were prepared from a marrow suspension secured by flushing the long bones of the hind limbs with a small volume of 0.9% saline solution. Following this the remainder of the marrow suspension was washed by alternately centrifuging slowly, decanting, etc. (× 3). The DNA was then isolated from the packed marrow cells by strong salt extraction (10% NaCl).

Since the isolation of β-amino isobutyric acid (Gerber et al., '60b) from the urine was not uniformly satisfactory in all samples, all radioactivity, which is not volatile upon air drying at room temperature (i.e., all radioactivity except tritium oxide), was determined instead. Three samples of urine of different size corresponding to a dry weight of less than 100 µg were dried on stainless steel planchets and assayed for radioactivity. This procedure was used to insure that the radioactive determinations were carried out at infinite thinness. β-Amino isobutyric acid could be isolated in about one-third of the samples collected. In these instances, the specific activity of β-amino isobutyric acid paralled the nonvolatile radioactivity. Radioactivity measurements on these as well as all other samples were carried out in a windowless gas-flow counter in the proportional region using methane. Since the total amount of urine excreted by the hamsters could not be determined with certainty, the radioactivity was related to the concentration of urinary creatinine (Peters et al., '46).

Samples of skeletal muscle (M. pectoralis major, M. rectus abdominus, M. adductor, M. Psoas, M. masseter, diaphragm) as well as heart, kidney, liver, spleen, duodenum, testis and uterus were fixed in glacial acetic acid/absolute methanol (1:3). Smears of bone marrow were prepared and then fixed in 1% acetic acid.

The tissues were embedded in paraffin at 60°C and after sectioning to a thickness of 7 µ, autoradiographs were prepared by conventional methods (Messier et al., '59), using liquid NTB3 Emulsion at 43°C. In the case of bone marrow, autoradiography was carried out directly on the fixed smears. The slides were exposed for 30 days in sealed plastic slide boxes containing anhydrous calcium sulfate and then processed in D19 developer. Distilled water was used as a stop bath. The slides were stained with Lillie-Mayer acetic acid alum hematoxylin (Lillie, '54) and mounted in polyvinylpyrrolidone (Burstone, '57).

Differentiation of cells in bone marrow was carried out on the basis of the following criteria, information being secured by the use of both phase and conventional microscopy.

A. Myeloid elements: Large cells without specific granules whose nuclei are large and exhibit a fine chromatin pattern are called myeloblasts. Cells with specific but incomplete granulation, nuclei showing small indentations and coarse chromatin patterns, are called myelocytes. Cells exhibiting a full complement of granules, nuclei which are extended, segmented, and contain densely clumped chromatin, are called segmented granulocytes.

B. Erythroid series: Small cells (8 to 12 µ) with a heavily basophilic cytoplasm, densely clumped chromatin, and a ratio of nucleus to cytoplasm of about 1:1 are called basophilic normoblasts. Cells of the same relative size but with a markedly less basophilic cytoplasm and markedly clumped and pycnotic-appearing nuclei are called polychromatic normoblasts. Cells (8 to 10 µ) whose cytoplasm lacked basophilia and whose nuclei are shrunken and markedly pycnotic are called orthochromatic normoblasts.

RESULTS

Radioactivity of isolated DNA and radioactivity excreted in urine. Table 1 shows data on the specific activity of DNA isolated from intestine, muscle, testis and bone marrow. As far as the intestine is concerned, no significant differences among vitamin E-supplemented, vitamin E-deficient and previously deficient, vita-

min E-treated hamsters were observed. The specific activity of DNA decreased rapidly with time during the initial period and then somewhat more slowly thereafter.

The specific activity of testicular DNA decreased at about the same rate in all three groups of animals although minor differences were observed in the initial specific activities of DNA isolated from testis. In muscle the specific activity of DNA was significantly higher initially and declined more rapidly with time in vitamin E-deficient hamsters than in vitamin E-supplemented or previously deficient, vitamin E-treated hamsters. The specific activity of DNA isolated from bone marrow was higher initially and declined more rapidly in vitamin E-deficient hamsters than in vitamin E-supplemented or previously deficient, vitamin E-treated hamsters.

The nonvolatile radioactivity excreted per milligram of creatinine in the urine was not significantly different in the three groups of hamsters (table 2). The values for the amount of radioactivity excreted per milligram of creatinine decreased rapidly with time and the resultant activity-time curve was similar to that of β-amino isobutyric acid excreted in rats after injection of H^3-thymidine (Gerber et al., '60c).

Autoradiography

Intestine. The mucosa and submucosa of the intestine of vitamin E-supplemented, vitamin E-deficient and previously deficient, vitamin E-treated hamsters had a normal histological appearance and labeling pattern (Messier et al., '60). On the first day after injection of H^3-thymidine, labeled cells were observed mainly at the bottom of the crypts of Lieberkühn

TABLE 1

Specific activity of deoxyribonucleic acid isolated from various organs of vitamin E-supplemented, vitamin E-deficient, and previously deficient, vitamin E-treated hamsters at different time intervals after injection of H^3-thymidine

	Specific activity														
	Muscle			Bone marrow			Testis			Intestine			Liver		
Days after injection	Vitamin E-supplemented	Vitamin E-deficient	Vitamin E-treated[1]	Vitamin E-supplemented	Vitamin E-deficient	Vitamin E-treated	Vitamin E-supplemented	Vitamin E-deficient	Vitamin E-treated	Vitamin E-supplemented	Vitamin E-deficient	Vitamin E-treated	Vitamin E-supplemented	Vitamin E-deficient	Vitamin E-treated
	count/min/μg DNA			*count/min/μg DNA*			*count/min/μg DNA*			*count/min/μg DNA*			*count/min/μg DNA*		
1	3.0[2,3]	6.5	2.3	11	18	10	4.3	4.5	3.1	30	22	23	2.7	2.9	3.1
3	2.7	5.6	2.0	7.5	10	7.6	3.6	3.6	2.3	13	12	15	2.8	2.7	3.0
7	2.9	2.6	2.4	2.5	3.2	2.0	3.3	3.4	2.0	6.0	3.8	3.1	2.6	2.8	2.7
30	2.2	1.9											2.4	2.2	2.4

[1] Vitamin E-treated indicates previously deficient, vitamin E-treated.
[2] Each value is the average of two animals.
[3] The standard error of the values presented is in the order of ± 15%.

TABLE 2

Nonvolatile radioactivity[1] excreted in the urine of vitamin E-supplemented, vitamin E-deficient and previously deficient, vitamin E-treated hamsters

	Radioactivity			
	1 Day	2 Days	3 Days	4 to 5 Days
Vitamin E-supplemented	180 ± 20[2]	15.0[3]	3.7	1.2
Vitamin E-deficient	208 ± 36	18.5	3.5	0.75
Deficient, vitamin E-treated	146 ± 16	16.0	2.9	1.2

[1] Nonvolatile radioactivity is expressed in counts/minute/microgram of creatinine excreted. The urine was collected over 24-hour intervals except on the fourth and fifth day when the collection period was 48 hours.
[2] Standard error of the mean.
[3] On the second to fifth day the standard error was approximately ± 15%.

(fig. 1). Sparse labeling occurred in the glands of Brünner. Epithelial cells of the villi were not labeled, but lymphatic nodules present in the sections showed intense labeling. Labeled cells were present in the submucosa and many of these labeled cells could be identified as macrophages and fibroblasts.

On the third day after injection of H^3-thymidine, labeled epithelial cells were found in the crypts and in the mid-region and lower third of the villi (fig. 2). The

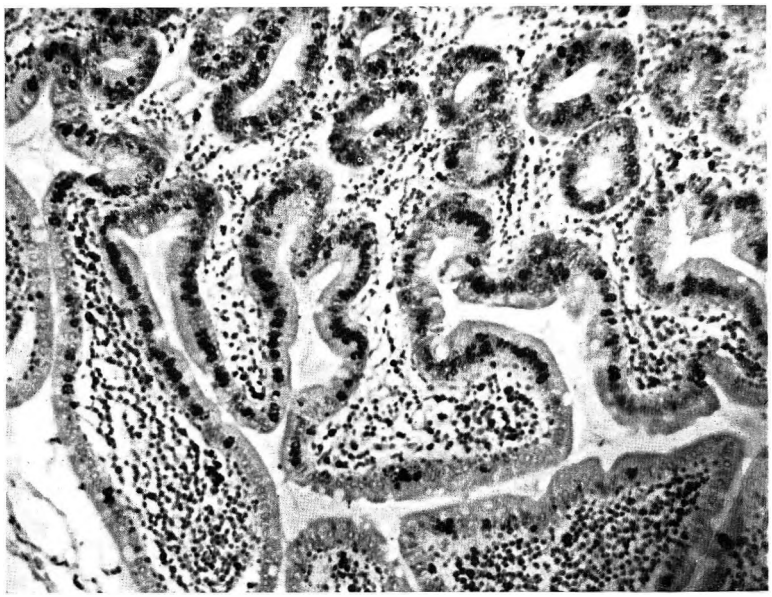

Fig. 1 Small intestine of vitamin E-deficient hamsters one day after injection of H^3-thymidine. Note the heavy labeling of the epithelium in the crypts of Lieberkühn and the comparative lack of label in the epithelium covering the villi.

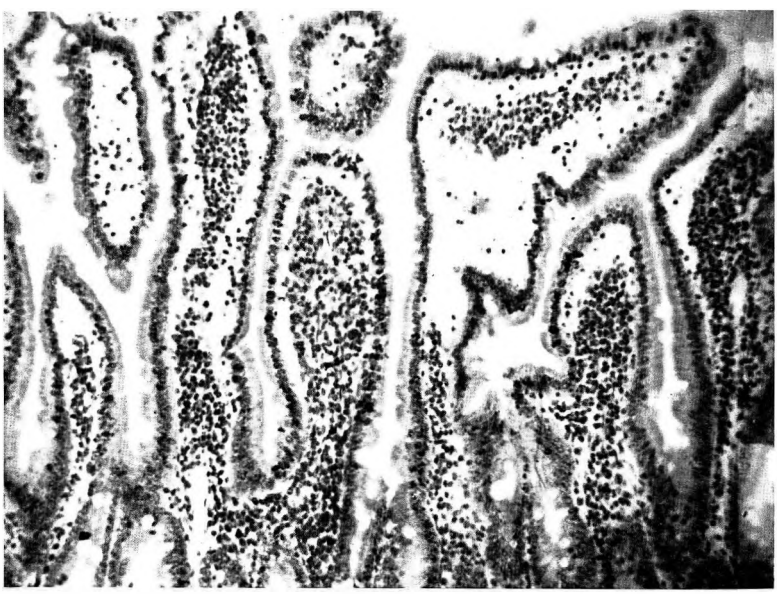

Fig. 2 Small intestine of vitamin E-deficient hamsters three days after injection of H^3-thymidine. Compare labeling pattern with that in figure 1.

number of labeled cells in the lymphatic nodules had decreased considerably. The labeling pattern in the submucosa was unchanged. Seven days after injection of H^3-thymidine, labeled epithelial cells were often found at the tip of the villi. At the bottom of the crypts a few labeled cells with relatively low grain counts were also observed. Lymphocytic elements were no longer labeled but labeled macrophages were abundant in the submucosa. Thirty days after injection of H^3-thymidine no labeling was found in the epithelium. Labeling was observed, however, in the glands of Brünner and in macrophages of the submucosa.

Testes. The testes of vitamin E-supplemented and vitamin E-deficient animals were morphologically normal. Unfortunately, in the latter animals the level of vitamin E depletion was sufficient to produce early muscle lesions but insufficient to induce clear-cut injury of the germinal epithelium; on the other hand, the hamsters selected for the previously deficient vitamin E-supplemented group proved to have suffered mild testis damage as indicated by early reparative response to therapy as described by Mason and Mauer.[1] Probably because of the marginal character of the testes damage, it was not possible to demonstrate by the procedures used, any significant difference in the pattern of labeling in the testes of the three different groups. The testes did, however, exhibit a characteristic pattern of labeling which depended on the time elapsed after injection of H^3-thymidine. On the first day after injection of H^3-thymidine, the label was found almost exclusively in the spermatogonia or resting spermatocytes, or both (fig. 3). These labeled cells were present in variable numbers in the seminiferous tubules. Seven days after the injection, many primary and some secondary spermatocytes as well as some young spermatids were also labeled (fig. 4). A few labeled spermatogonia were found in tubules which did not contain any labeled spermatocytes. At all time intervals studied an occasionally labeled Leydig cell, macrophage or fibrocyte were seen in the interstitium.

Spleen. One day after injection of H^3-thymidine, labeled cells were found in about equal numbers scattered throughout the red and white pulp of the spleen of

[1] A preliminary report of these studies has been published: Mason, K. E., and S. I. Mauer 1957 Reversible testis damage in the vitamin E-deficient hamster. Anat. Rec., *127*: 329 (abstract).

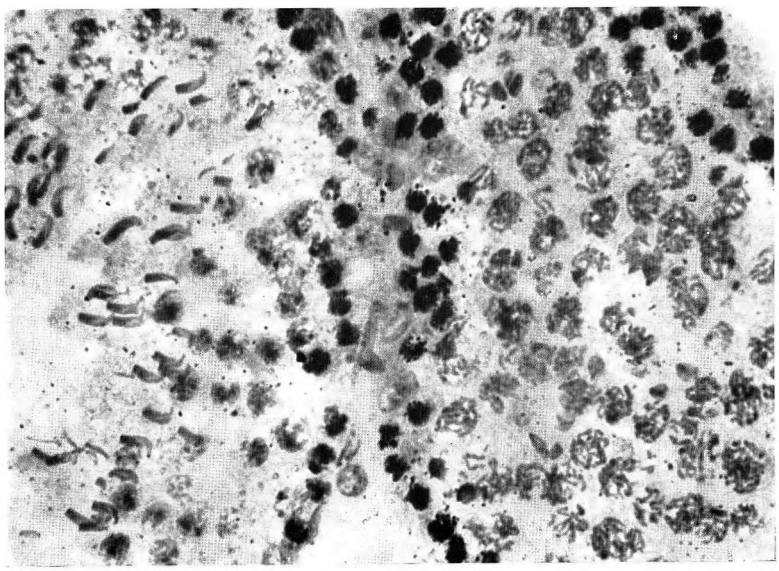

Fig. 3 Testis of vitamin E-deficient hamster one day after injection of H^3-thymidine. Note the heavily labeled spermatogonia and resting spermatocytes.

vitamin E-supplemented and previously deficient, vitamin E-treated hamsters. The labeled cells in the red pulp were consistently found in clusters among scattered islands of intensely basophilic cells. In the white pulp, lymphocytes were labeled. A few germinal centers showed clusters of labeled cells. In vitamin E-deficient hamsters markedly more labeled cells were found in the white pulp than in vitamin E-supplemented or previously deficient, vitamin E-treated hamsters (figs. 5, 6). The cell types labeled in the white pulp of vitamin E-deficient animals appeared to be

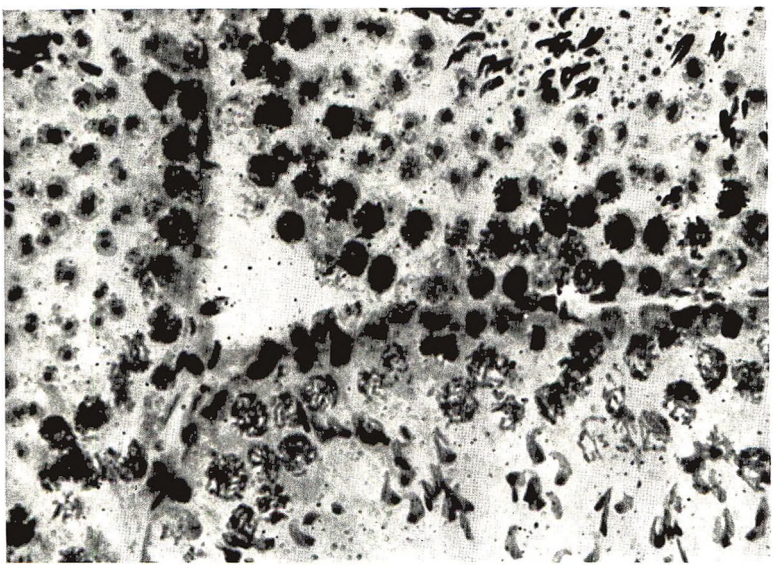

Fig. 4 Testis of vitamin E-deficient hamster 7 days after injection of H^3-thymidine. At this time mainly spermatocytes are labeled.

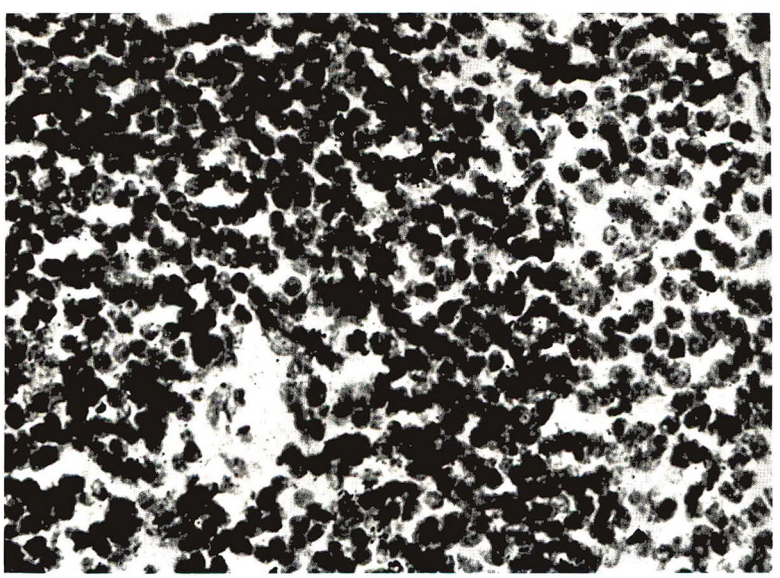

Fig. 5 Spleen of vitamin E-supplemented hamster one day after injection of H^3-thymidine.

the same as those labeled in the vitamin E-supplemented hamsters.

Three days after the injection of H^3-thymidine, the differences between vitamin E-deficient and vitamin E-supplemented hamsters were no longer apparent and labeled cells were found uniformly distributed throughout the red and while pulp. The number of labeled cells, however, had decreased significantly as compared with that on the first day after injection. Still fewer labeled cells were found at 7 days and most of these cells were macrophages or reticular cells, or both. At 30 days, labeled cells had almost completely disappeared except for either a few labeled macrophages or reticular cells, or in some instances, both.

Heart. Cardiac muscle of vitamin E-deficient hamsters appeared normal. Labeling was restricted to fibroblasts, macrophages and leucocytes but cardiac muscle cell nuclei were not labeled.

Skeletal muscle. The skeletal muscle of hamsters deficient in vitamin E showed the typical histopathology of vitamin E deficiency (West and Mason, '55 and '58). Focal degeneration of myofibrils and extensive coagulation necrosis were seen. Infiltration of the areas of coagulation by macrophages and leucocytes was observed, converting the lesion into a highly cellular mass (figs. 7, 8 and 9). Muscle nuclei arranged in chain-like rows were seen frequently (fig. 10). These "rowed nuclei" resembled somewhat nuclei of embryonic muscle but indications of degeneration (e.g., vacuolization) were present.

Following vitamin E therapy all evidence of necrosis as well as the areas of infiltration disappeared within a few days. Thus, three days after the administration of α-tocopherol, lesions were rarely seen and, after this time, were no longer apparent. However, "nuclear rowing" was still very much in evidence. No degenerative changes were observed in the skeletal muscle of vitamin E-supplemented hamsters but "nuclear rowing" was occasionally found.

Labeling in the muscle of vitamin E-deficient animals was confined essentially to the areas of degenerative lesions and the labeled cells were mainly macrophages, leucocytes and fibroblasts (figs. 7, 8 and 9). The number of labeled cells in the lesions decreased markedly three to seven days after injection of H^3-thymidine as compared with the first day. In vitamin E-supplemented and previously defi-

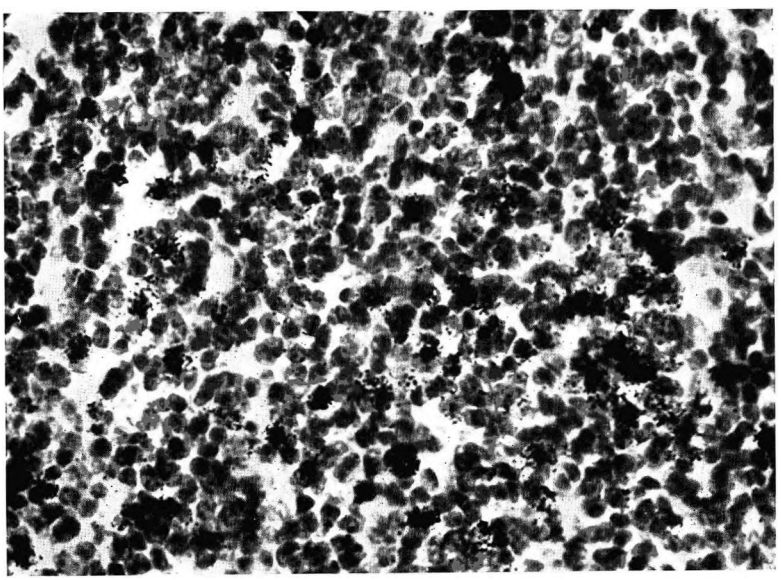

Fig. 6 Spleen of vitamin E-deficient hamster one day after injection of H^3-thymidine. Note the heavy labeling in the white pulp as compared with figure 5.

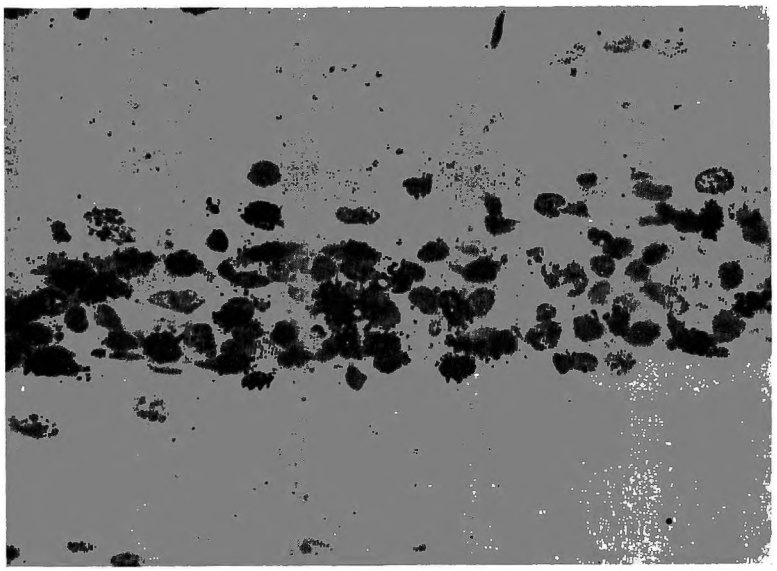

Fig. 7 Vitamin E-deficient muscle one day after injection of H^3-thymidine. The lesion contains heavily labeled cells (macrophages and leucocytes).

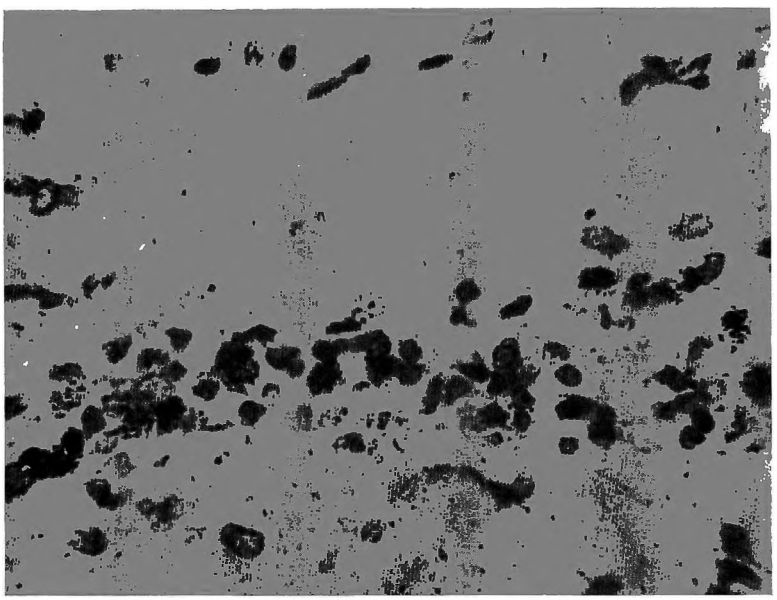

Fig. 8 Vitamin E-deficient muscle three days after injection of H^3-thymidine. Note the decrease in the number of labeled cells in the lesion.

cient, vitamin E-treated animals, labeling was also observed in fibroblasts and macrophages. However, because of the absence of lesions a considerably smaller number of cells per field of view was labeled than in the vitamin E-deficient muscle.

Muscle nuclei were labeled very rarely, if at all, in vitamin E-supplemented, previously deficient, vitamin E-treated and vitamin E-deficient hamsters. In more than 100 sections examined, only two muscle nuclei showed labeling and here the iden-

tification was doubtful. More than 5,000 nuclei arranged in "nuclear rows" in the skeletal muscle of all three groups of hamsters were counted, but only one cell nucleus of doubtful identification was labeled.

Bone marrow. The frequency of labeled cells in bone marrow as a function of time after injection of H^3-thymidine in vitamin E-supplemented, previously deficient, vitamin E-treated and vitamin E-deficient hamsters is shown in table 3. Each value in the table is based on a total count of at least 2,000 cells. On the first day after the injection of H^3-thymidine, the number

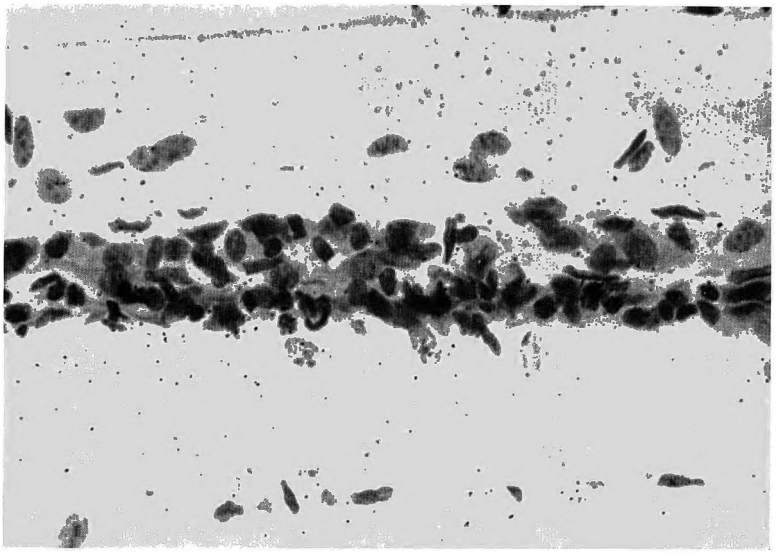

Fig. 9 Vitamin E-deficient muscle 7 days after injection of H^3-thymidine. Note that the lesion contains very few labeled cells as compared with figure 7.

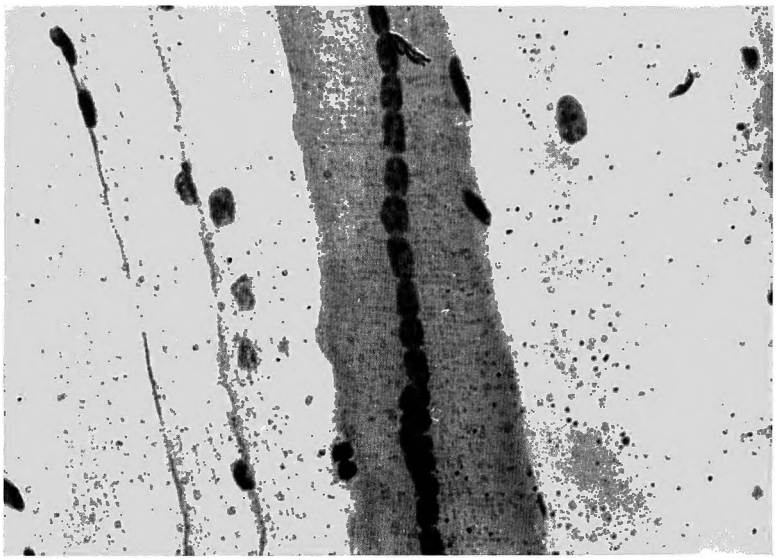

Fig. 10 "Nuclear rowing" in the muscle of vitamin E-deficient hamsters. No labeling could be found in nuclei present in these rows.

TABLE 3

Number of cells labeled and differential count in the bone marrow of vitamin E-supplemented, vitamin E-deficient and previously deficient, vitamin E-treated hamsters

	Number of cells[1] labeled/1,000 cells			Differential counts[2] on the first day after injection of H^3-thymidine																	
				Myeloid elements									Normoblasts								
				Myeloblasts			Myelocytes			Segmented granulocytes			Basophilic			Polychromatic			Orthochromatic		
	1 Day	3 Days	7 Days	A[3]	B[4]	C[5]	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Vitamin E-supplemented	65	34	7	5	2	16	44	24	13	33	62	4	14	3	6	3	6	4	1	4	2
Vitamin E-deficient	110	36	4	13	6	26	28	31	10	36	54	8	19	6	11	4	2	20	0	1	–
Previously deficient vitamin E-treated	72	27	7	6	2	25	31	28	8	35	53	5	16	6	7	13	8	12	0	4	–

[1] In each case, at least 2,000 cells were counted in 4 smears.
[2] Total count and differentiation of at least 400 labeled cells/smear. The degree of error in these determinations is undoubtedly high due to the technique of making and staining the smears. The progression, however, is that which one would expect in the light of other data (Dinning et al., '55, '57).
[3] A indicates percentage of labeled cells of a particular type/total number of all counted labeled cells.
[4] B indicates percentage of cells/total number of all cells (labeled and unlabeled) counted (all types).
[5] C indicates percentage of labeled cells/total cells (labeled and unlabeled) of a particular type.

of labeled cells was increased in vitamin E-deficient hamsters as compared with those of previously deficient, vitamin E-treated or vitamin E-supplemented animals, but was unchanged at the later time points. The bone marrow of vitamin E-deficient hamsters exhibited a relative increase in number of myeloblasts and basophilic normoblasts and a decrease in polychromatic and orthochromatic normoblasts (cell types as defined herein) (table 3). The proportion of labeled cells of a particular type one day after injection of H^3-thymidine was almost always greater in the marrow of vitamin E-deficient hamsters than in that of vitamin E-supplemented hamsters indicating that bone marrow cells were replaced more rapidly in the vitamin E-deficient animals.

DISCUSSION

Numerous studies have demonstrated that DNA is metabolically stable in the resting cell and is synthesized only by actively dividing cells (Taylor, '57). If H^3-thymidine is administered to an animal, only cells that are preparing for division and are, therefore, actively synthesizing DNA, will incorporate the labeled precursor into DNA. Incorporation of H^3-thymidine into DNA subsequent to a single administration of the precursor is completed within a few hours (Nygaard et al., '59) and the specific activity of DNA 24 hours after injection of H^3-thymidine permits estimation of the amount of DNA synthesized during the period of availability of H^3-thymidine for DNA synthesis.

Later the specific radioactivity of DNA decreases with time owing to dilution by newly synthesized, unlabeled DNA. Thus, the decrease in specific activity of DNA also reflects the rate of DNA synthesis. However, unlike the incorporation of labeled precursor into DNA, the replacement of labeled DNA by newly synthesized unlabeled DNA is not influenced by changes in the metabolic pools of precursors of DNA, unless the concentrations of these precursors limit the rate of DNA biosynthesis.

It has been reported that the incorporation of formate into DNA of muscle and bone marrow of vitamin E-deficient animals is increased whereas in other organs

no differences are apparent (Dinning et al., '55, '58). On the other hand, incorporation of glycine-1-C^{14} into DNA of muscle and bone marrow of vitamin E-deficient animals is approximately the same as in normal animals, while it is decreased in many other organs (Dinning et al., '55). Our data indicate that incorporation of H^3-thymidine followed the same pattern as found for incorporation of formate, since higher specific activities were observed in DNA isolated from muscle and bone marrow of vitamin E-deficient hamsters one day after injection of H^3-thymidine. This similarity is not unexpected because formate can function as a direct precursor of the methyl group of thymidine (Elwyn, '50), whereas the carbon atom of the carboxyl group of glycine behaves in quite a different manner metabolically.

Since our data indicate that the incorporation of H^3-thymidine into DNA as well as the turnover of DNA is altered in the muscle and bone marrow of vitamin E-deficient hamsters, it may be concluded that these changes are due to alterations in the biosynthesis of the polymeric molecule of DNA rather than to effects on the metabolism of DNA precursors per se. Since the interpretation of the data based on the autoradiographs differs in the case of muscle and bone marrow, these two organs will be treated separately for further discussion.

Muscle. As set forth previously only the skeletal muscle of vitamin E-deficient hamsters showed degenerative lesions with extensive cellular infiltration. Many of the cells infiltrating these lesions were labeled after injection of H^3-thymidine and the number of labeled cells decreased with time after injection. Since muscle cell nuclei were labeled very rarely, if at all, it appears that the rapid replacement and division of cells in and around the degenerative lesions in vitamin E-deficient muscle is responsible for the observed increase in DNA turnover in the deficient hamster.

An interesting aspect of the histopathology of vitamin E-deficiency is the phenomenon of "nuclear rowing" observed in deficient skeletal muscle. It has been suggested that "nuclear rows" represent either a reaction to sublethal and reversible injury or a structural response related to a reparative or regenerative effort by muscle (West and Mason, '55, '58). The question has been raised as to whether these muscle nuclei are merely sarcolemmic nuclei which have been displaced from their normal position in the fiber or are nuclei which have undergone mitotic or amitotic division, or both. The autoradiographs prepared from muscle one, three, seven and 30 days after injection of H^3-thymidine demonstrate that "rowed nuclei" are not labeled to a significant extent. Thus, DNA synthesis and, therefore, cell division must have occurred only rarely during the time period studied. While the possibility that these cells may have undergone mitotic or amitotic division at a rapid rate at some earlier stage of vitamin E-deficiency cannot be excluded, this possibility appears unlikely because no histological evidence for cell division during the early stages of vitamin E-deficiency has ever been found (West and Mason, '55, '58). Thus, it appears likely that "rowed nuclei" represent displaced sarcolemmic nuclei which may have undergone differentiation to a myoblastic stage, but do not undergo cell division. Myoblastic nuclei have been reported to divide only rarely during embryonic development (Stockdale et al., '61). However, after direct injury, muscle cell nuclei have been found labeled (Bintliff et al., '60). An estimate of the average life time of a cell nucleus of hamster muscle can be obtained if one assumes that the cells divide randomly and that the period after injection of thymidine during which the isotope concentration is sufficient for cell labeling is about three hours. Since less than one in 5,000 nuclei was found to be labeled, the average life time of such a nucleus would be more than 800 days.

Bone marrow. In addition to changes in nucleic acid metabolism, vitamin E-deficient animals display granulocytosis and anemia (Dinning et al., '55, '57). Our findings confirm the observation that DNA synthesis and cell renewal are accelerated in the bone marrow of vitamin E-deficient animals. The autoradiographic and histological data indicate that almost all types of bone marrow cells are replaced more rapidly in vitamin E-deficient hamsters.

The mechanism responsible for this increased replacement of granulocytes is unclear although it may be related to a stimulatory effect on the marrow produced by noxious agents liberated from the lesions found in vitamin E-deficient muscle.

It is reasonable to assume that the increase in replacement of erythroid elements in the bone marrow of vitamin E-deficient hamsters is related to the anemia of vitamin E deficiency. Following vitamin E therapy the labeling patterns of cells of the erythroid as well as of the myeloid series approximate but are not entirely like the pattern observed in vitamin E-supplemented hamsters.

Other organs. No difference in DNA metabolism between vitamin E-deficient, previously deficient, vitamin E-treated or vitamin E-supplemented hamsters was observed in liver, intestine or testis. The time course of labeling found in the intestine followed that described in other animals (Messier et al., '60). On the basis of histological findings in the testes, which indicated no obvious differences in labeling pattern with time, it was concluded that the maturation of spermatogonia in the vitamin E-deficient hamsters exhibiting early muscle lesions was normal.

The autoradiographic studies show that one day after injection of H^3-thymidine the white pulp of the spleen of vitamin E-deficient hamsters contains markedly greater numbers of labeled cells as compared with that of the controls. It appears likely that these changes reflect differences in DNA metabolism of leucocytes (nongranular series) similar to those found in the bone marrow.

In summary, the changes in the metabolism of DNA found in skeletal muscle of vitamin E-deficient hamsters are related to altered cellular composition resulting from the presence of degenerative lesions with infiltration of the affected areas by inflammatory cells. Changes in the bone marrow result from an accelerated turnover of cells which are normally present in bone marrow.

SUMMARY

Vitamin E-supplemented, vitamin E-deficient and previously deficient, vitamin E-treated hamsters were injected intraperitoneally with H^3-thymidine. Animals from each group were killed at one, three, 7 and 30 days after injection of H^3-thymidine. Autoradiographs were prepared from sections of various tissues after which the slides were stained with acid-alum hematoxylin. The DNA was isolated from liver, intestine, muscle, testis and bone marrow and assayed for radioactivity. In addition, nonvolatile radioactivity excreted in the urine was also determined.

DNA synthesis and turnover were increased in the muscle and bone marrow of vitamin E-deficient hamsters as compared to vitamin E-supplemented or previously deficient, vitamin E-treated hamsters. No significant differences in DNA turnover of liver, testis, or intestine were observed among vitamin E-supplemented, vitamin E-deficient and previously deficient, vitamin E-treated hamsters. Autoradiographic data support this observation and provide evidence for the view that the presence of a large number of inflammatory cells, which infiltrate the degenerative lesions, present in vitamin E-deficient skeletal muscle is responsible for the observed, accelerated DNA metabolism. Incorporation of H^3-thymidine into DNA as well as the turnover of DNA also is increased in the bone marrow of vitamin E-deficient hamsters and it appears that all types of hematopoietic cells participate in the increased DNA metabolism. The significance of this finding with respect to the granulocytosis and anemia present in vitamin E deficiency is discussed.

ACKNOWLEDGMENT

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Effect of Methionine and Riboflavin on Free Amino Acids, Riboflavin and Lipids in Selected Rat Tissues[1]

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The addition of methionine to diets adequate or inadequate in this amino acid resulted in decreased levels of free valine in plasma of chicks (Richardson et al., '53a). The concentration of valine in plasma of rats increased when oxidized casein was added to a ration which contained peanut meal to provide a methionine imbalance (Sauberlich, '56). A recent report (Sauberlich, '61a) showed that the addition of excess amino acids to rations containing casein increased the plasma level of the amino acid added to the diet and depressed growth. These effects depended on the level and kind of protein used in the basal diet and were not related directly to the plasma concentration of a particular amino acid.

A correlation between methionine intake and concentration of riboflavin in liver of rats (Riesen et al., '46) and an inverse relationship between dietary riboflavin and the level of free methionine, valine and tryptophan in plasma of chicks fed a partially purified diet have been reported (Richardson et al., '53b). Other workers have observed that methionine supplementation of cholesterol-containing diets decreased liver cholesterol in the rat (Channon et al., '38; Okey and Lyman, '54). This study reports the effect of varying levels of L-methionine and riboflavin upon the concentration of free arginine, lysine, methionine, tryptophan and valine in plasma and riboflavin-containing coenzymes, cholesterol and total lipids in liver of rats fed rations containing casein.

EXPERIMENTAL

Weanling male rats obtained from Sprague-Dawley were allotted according to litter and body weight to 6 groups of 12 animals/group. Two groups of rats were each fed one of the following: (1) riboflavin-deficient basal ration; (2) basal ration supplemented with 0.2% of L-methionine; (3) basal ration supplemented with 2.0% of L-methionine. Six days per week, one set of three groups received daily 10 μg of riboflavin in aqueous solution and the other set of three groups received 100 μg. The methionine supplements were added at the expense of sucrose to the basal ration which contained 18% of casein and has been described (Harrill et al., '59). The rats were housed in individual wire-bottom cages and food and distilled water were provided ad libitum during the 4-week experimental period.

Blood was collected from decapitated rats in centrifuge tubes which contained 0.2 mg of sodium heparinate/ml of blood (ICNND, '57). Equal volumes of plasma from 4 rats in each group were pooled for the determinations with the exception of the group fed 18% of casein supplemented with 10 μg of riboflavin in which one rat died. Protein-free filtrates were prepared (Hier and Bergeim, '45) and analyzed microbiologically for 5 amino acids (Henderson et al., '48). The organisms were the same as those used by Richardson et al. ('53b).

The liver was frozen with solid carbon dioxide and stored at −20°C until analyses could be completed for flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) plus free riboflavin by the fluorometric method of Bessey et al. ('49).

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Liver was analyzed for cholesterol by the revised method of Sperry and Webb ('50) and total lipids of liver were measured by the method of Bloor ('28) as modified by Okey and Lyman ('54).

RESULTS

Free amino acids in plasma. The mean concentrations of free arginine, lysine, methionine, tryptophan and valine in plasma of rats fed the experimental diets are shown in table 1. The addition of 0.2% of methionine to the basal ration significantly decreased the level of plasma valine in animals fed 10 μg of riboflavin ($P < 0.05$). The decrease observed at the higher level of riboflavin was not significant. The lysine level increased at the lower level of riboflavin intake with 0.2% of methionine but the difference was not significant. No significance is associated with the small increases in arginine or methionine or the decreases observed in tryptophan.

The higher level of methionine (2.0%) greatly increased the plasma methionine at both levels of riboflavin intake. The differences were significant at the 1% level. The lysine concentration in animals fed 10 μg of riboflavin significantly increased above that of the mean value for similar animals fed the basal ration ($P < 0.05$). The mean changes in arginine and tryptophan for the diet that contained 2.0% of added methionine were in general similar to those observed when the methionine intake was increased by 0.2%. The changes were not significant. The data also show that the high-methionine diet resulted in a further decrease of valine in animals fed 100 μg of riboflavin but the difference was not significant.

The concentration of valine was greater in animals fed 100 μg of riboflavin as compared with corresponding animals fed 10 μg of the vitamin with 18% of casein ($P < 0.01$) or 0.2% of methionine ($P < 0.05$). The only significant effect of riboflavin on plasma methionine was observed in animals fed 2.0% of methionine. The level increased in animals fed 100 μg of the vitamin ($P < 0.01$). Plasma arginine, lysine and tryptophan were not affected significantly by dietary riboflavin.

Growth. A summary of the growth data (table 1) shows that the addition of 0.2% of methionine to the basal ration resulted in increased growth of animals fed 100 μg of riboflavin but had no effect on growth of animals fed 10 μg of the vitamin. Supplementation of the same ration with 2.0% of methionine markedly inhibited growth of animals fed either level of riboflavin. The spleens of the animals fed excessive methionine were dark, and riboflavin deficiency symptoms were observed in the animals fed 10 μg of riboflavin. The higher level of riboflavin increased the growth rate of animals on each level of methionine intake. Although the intake of 10 μg of riboflavin produced typical deficiency symptoms in only a few animals fed rations containing 18% of casein or 18% of casein plus 0.2% of methionine, one animal fed 18% of casein and no added methionine died on the twentieth day of the experiment.

Liver riboflavin. A comparison of the mean values for riboflavin-containing coenzymes (table 2) shows that the level of

TABLE 1

Effect of riboflavin and methionine supplementation on weight gain and free amino acids in plasma of rats fed a diet containing 18% of casein

Supplementation		Weight gain	Free amino acids[1]				
L-Methionine	Riboflavin[2]		Arginine	Lysine	Methionine	Tryptophan	Valine
%	μg	gm	μg/ml	μg/ml	μg/ml	μg/ml	μg/ml
—	10	90	25.7±4.1	60.5±4.8	10.2± 1.9	28.4±3.0	35.9±1.3
—	100	162	25.5±4.5	70.2±2.5	9.9± 1.2	25.0±2.3	49.0±0.5
0.2	10	90	29.5±5.6	71.9±2.0	14.2± 0.5	23.6±4.8	27.0±2.3
0.2	100	182	26.1±2.7	68.3±9.4	14.6± 1.9	22.9±4.8	41.2±3.8
2.0	10	47	34.9±3.1	78.1±3.7	141.1±21.8	23.0±3.7	40.0±1.7
2.0	100	74	31.9±0.7	67.7±7.4	201.1±10.0	17.1±3.2	38.0±4.2

[1] Mean value of three pooled samples ± standard error of the mean.
[2] Riboflavin supplement was given six times/week.

TABLE 2

Effect of riboflavin and methionine supplementation on riboflavin and derivatives, cholesterol and total lipids in liver of rats fed a diet containing 18% of casein

Supplementation		Riboflavin and derivatives		Total cholesterol[1]	Total lipids[1]
L-Methionine	Riboflavin	Free +FMN[1]	FAD[1]		
%	μg	μg/gm	μg/gm	mg/gm	mg/gm
—	10	2.16 ± 0.41	13.67 ± 0.72	2.18 ± 0.03	35.5 ± 1.2
—	100	4.24 ± 0.37	21.51 ± 1.21	1.95 ± 0.04	33.5 ± 0.6
0.2	10	2.12 ± 0.23	14.60 ± 0.83	2.08 ± 0.03	34.8 ± 1.2
0.2	100	4.60 ± 0.26	24.85 ± 1.67	1.91 ± 0.05	33.3 ± 0.7
2.0	10	2.71 ± 0.32	13.33 ± 0.66	2.00 ± 0.05	35.7 ± 1.4
2.0	100	4.90 ± 0.45	27.75 ± 2.22	1.85 ± 0.04	35.7 ± 1.0

[1] Mean ± standard error of the mean.

methionine intake had no significant effect on the concentration of the coenzymes in liver of animals fed 10 μg of riboflavin. At the higher level of riboflavin intake (100 μg) the level of FAD in livers of animals fed 2.0% of methionine was significantly greater ($P < 0.05$) than that of animals fed the basal ration. Increasing the riboflavin intake significantly increased the mean free riboflavin plus FMN or FAD in liver of animals fed either ration ($P < 0.01$).

Liver cholesterol. At each level of riboflavin intake the mean concentration of cholesterol in liver decreased slightly with increased levels of dietary methionine (table 2). The differences observed at the higher level of riboflavin supplement were not significant. At the lower level of riboflavin (10 μg) the difference between the mean value for animals fed the basal ration and that of animals fed 0.2% of methionine was significant ($P < 0.05$). The difference between the averages for animals fed the basal or 2.0% of methionine was significant at the 1% level. The higher level of riboflavin at each level of methionine significantly decreased the level of cholesterol in animals fed the basal ration or 0.2% of methionine ($P < 0.01$) or 2.0% of methionine ($P < 0.05$).

Total lipids in liver. Table 2 shows the mean concentration of total lipids in liver of the experimental animals. The slight increase in concentration of total lipids in liver of animals fed 2.0% of supplemental methionine at the higher level of riboflavin was the only significant difference due to methionine intake ($P < 0.05$). No significant effect of dietary riboflavin was observed.

DISCUSSION

The increased concentrations of methionine in plasma of animals which resulted from the feeding of 2.0% of methionine is in agreement with reports by other workers (Hier, '47; Richardson et al., '53a; Sauberlich, '61a). The lower level of plasma methionine observed in animals fed 2.0% of added methionine and 10 μg of riboflavin as compared with that of corresponding animals fed 100 μg of the vitamin may have been due to lowered food intake. Richardson et al. found that a basal diet low in riboflavin resulted in increased levels of methionine, valine and tryptophan of chicks ('53b), and increased dietary methionine decreased plasma valine in chicks fed a ration which contained peanut meal ('53a). Results of the present study show that at the lower level of riboflavin the concentration of plasma valine decreased with 0.2% of methionine and lysine increased with 2.0% of the amino acid. An increase in valine due to increased riboflavin intake was observed in animals fed the basal or 0.2% of methionine.

These studies indicate that various factors such as amount and kind of protein, amino acid imbalance, vitamin intake and species difference may affect the level of free amino acids in plasma. The significance of these changes in the growing animal is unknown but additional knowledge with respect to deviation from a pat-

tern typical of an adequate nutrient intake should be useful in assessing metabolic disturbances.

The increased retention of total riboflavin in liver of animals fed 100 μg of riboflavin and supplemental methionine is in agreement with observations from other laboratories (Unna et al., '44; Riesen et al., '46). In addition this study showed that the increase was due almost entirely to FAD.

The concentration of liver cholesterol in animals fed 10 μg of riboflavin decreased when 0.2 or 2.0% of methionine was added to the basal ration. Channon et al. ('38) and Okey and Lyman ('54) reported that the feeding of methionine with cholesterol-containing diets decreased liver cholesterol. However, Roth and Milstein ('57) found that a supplement of 4.8% of DL-methionine, more than double the higher amount in this experiment, resulted in an increased retention of liver cholesterol.

Methionine supplementation appeared to have little effect on the concentration of total lipids in liver. Harper et al. ('54) found that the addition of 0.1% of DL-methionine to a ration that contained 9% of casein, and choline increased the fat content of the liver of young rats. The fat content was reduced by the addition of threonine to the ration. Brown and Allison ('48) used a ration which contained 12% of casein with 4.8% of DL-methionine and observed a decrease of total fat in liver of rats (weight not given). According to Roth and Milstein ('57) a similar ration produced no change in fatty acid content of liver of rats weighing 160 to 200 gm.

Relationships between dietary methionine and dietary glycine or arginine or both (Brown and Allison, '48; Van Pilsum and Berg, '50; Hardin and Hove, '51; Cohen et al., '58; Waterhouse and Scott, '61), fat (Roth and Milstein, '57; Fox et al., '59), cystine (Rama Rao et al., '61) and pyridoxine (DeBey et al., '52; Sauberlich, '61b) have been reported. It is recognized that the level of these nutrients which were held constant in the present experiment may have influenced the results.

SUMMARY

The effect of riboflavin and methionine supplementation of a riboflavin-deficient ration which contained 18% of casein, on the concentration of amino acids in plasma and riboflavin-containing coenzymes, cholesterol and total lipids in liver of rats was studied. The addition of 0.2% of L-methionine to a riboflavin-deficient basal ration which contained 18% of casein decreased the concentration of plasma valine in young rats fed 10 μg of riboflavin. A marked increase in plasma methionine was observed in animals fed 2.0% of methionine. A comparison of data for animals fed 2.0% of methionine and the basal ration showed an increase in plasma lysine for animals fed 10 μg of riboflavin.

Increasing the riboflavin intake from 10 to 100 μg increased plasma methionine in animals fed 2.0% of methionine. The higher level of riboflavin resulted in increased plasma valine in animals fed the basal or 0.2% of methionine.

Supplementation of the basal ration with 0.2% of L-methionine increased the growth rate of animals fed 100 μg of riboflavin above that of corresponding animals fed the basal ration but had no effect on animals fed 10 μg of the vitamin. The addition of 2.0% of L-methionine to the basal ration markedly decreased the growth rate of the animals fed either amount of riboflavin.

Increased methionine intake increased the total riboflavin retention in liver of animals fed 100 μg of riboflavin. The concentration of liver cholesterol decreased with increased riboflavin and decreased with increased methionine with one exception in animals fed 10 μg of the vitamin. Supplemental methionine or riboflavin had little effect on total lipids in liver.

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Some Effects of High and Low Sodium Intakes During Pregnancy in the Rat[1,2]

III. SODIUM, POTASSIUM AND WATER IN MATERNAL ADRENALS AND HEARTS

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The previous papers in this series presented data on the effects of a dietary sodium deficit and surfeit on food consumption, weight gain, reproductive performance, sodium and potassium balance, urinary excretion of sodium and potassium, hematocrit, hemoglobin and plasma proteins in the nonpregnant and pregnant rat (Kirksey and Pike, '62), and on the sodium and potassium levels of maternal plasma, muscle, bone and brain, fetal plasma, amniotic fluid, placenta and total fetus (Kirksey et al., '62). This report is concerned with the concentrations of sodium and potassium, and percentages of water in maternal adrenals and hearts.

EXPERIMENTAL PROCEDURE

The experimental design and the dietary treatment for the entire study were presented previously (Kirksey and Pike, '62).

At the end of a three-week period for nonpregnant rats, tissues were removed for analysis. At term for pregnant animals, the young were removed by abdominal section and maternal tissues to be analyzed were removed. Hearts and adrenals were blotted to remove excess blood and then weighed. Each pair of adrenals and each heart was dried to constant weight in a weighed silica crucible at 100 C. After obtaining dry weights, percentage of water was calculated. Samples were then ashed in a muffle furnace at 525C. Muffling time was 24 hours for adrenals and 48 hours for hearts. The ash was dissolved in 2 N HCl, heated, filtered through Whatman no. 40 filter paper and brought up to a volume of 10 ml for adrenals and 100 ml for hearts with distilled demineralized water. The percentage of transmittancies of sodium and potassium were determined in a Beckman DU Flame Photometer equipped with a photomultiplier attachment. Sodium was read at 589 mμ and potassium at 768. The method of bracketing was followed in reading all samples. All sodium and potassium values were calculated on the wet weight of the sample and expressed as milliequivalents per kilogram.

RESULTS AND DISCUSSION

The average weights of the adrenals of the nonpregnant and pregnant rats at the three levels of sodium intake are presented in table 1. The level of dietary sodium did not affect the average total weight of the adrenals of nonpregnant animals; nor did pregnancy have any significant effect on the weight of the adrenals. However, there was a significant interaction between pregnancy and low sodium leading to an increase in the absolute weight of the glands. When adrenal weight was calculated on the basis of 100 gm of body weight, in order to compensate for the differences in total body weight between pregnant and nonpregnant animals at the end of the experimental period, a significant decrease in both wet and dry weight due to pregnancy was apparent ($P = 0.01$).

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TABLE 1

Average adrenal weights of nonpregnant and pregnant rats

Groups	Dietary Na	Total wet weight	Weight/100 gm body weight	
			Wet	Dry
	mEq/100 gm	*mg*	*mg*	*mg*
Nonpregnant	1.4	59 ± 3.3[1]	22 ± 1.3	7 ± 0.4
	6.3[2]	60 ± 2.9	23 ± 1.2	7 ± 0.4
	54.8	58 ± 3.9	23 ± 1.3	7 ± 0.4
Pregnant	1.4	82 ± 8.0	28 ± 3.1	8 ± 0.7
	6.3[2]	54 ± 2.3	15 ± 0.5	4 ± 0.4
	54.8	52 ± 2.9	14 ± 0.6	4 ± 0.2

[1] Standard error of the mean.
[2] Control group.

TABLE 2

Average sodium, potassium and water content of adrenal glands of nonpregnant and pregnant rats

Groups	Dietary Na	Sodium	Potassium	Water
	mEq/100 gm	*mEq/kg*	*mEq/kg*	%
Nonpregnant	1.4	36.38 ± 0.82[1]	70.91 ± 1.52	69.97 ± 0.46
	6.3[2]	37.35 ± 0.86	68.90 ± 2.19	69.98 ± 0.46
	54.8	37.60 ± 1.20	67.99 ± 1.79	68.05 ± 0.90
Pregnant	1.4	31.13 ± 0.77	69.85 ± 2.53	71.93 ± 0.49
	6.3[2]	34.16 ± 0.60	68.10 ± 1.49	69.87 ± 0.83
	54.8	33.74 ± 0.74	64.91 ± 1.02	70.04 ± 0.57

[1] Standard error of the mean.
[2] Control group.

However, the interaction between pregnancy and low salt led to a highly significant increase in both the relative wet and dry weights ($P = 0.001$). This hypertrophic response did not occur as a result of either pregnancy or low dietary sodium alone, but was the result of a combination of the two. Other investigators have reported adrenal hypertrophy, specifically of the zona glomerulosa (Deane et al., '48; Cohen and Crawford, '62), and increased aldosterone production (Luetscher and Axelrad, '54; Eisenstein and Hartroft, '57) in sodium deficiency. Because during pregnancy there is an expansion of extra- and intracellular fluid volume, the need for increased sodium should be expected. The superimposition of a sodium deficiency upon the pregnant state would further the need for sodium conservation. The significance of the interaction between pregnancy and low sodium intake in producing adrenal hypertrophy reinforces our previous suggestion that a sodium deficiency was present in the pregnant group receiving the low sodium diet.

The sodium, potassium and water content of the adrenals are shown in table 2. The adrenal glands of all pregnant rats had a significantly lower ($P = 0.001$) concentration of sodium than those of nonpregnant animals. Although the level of dietary sodium had no significant effect on adrenal sodium concentrations in either the nonpregnant or pregnant groups, the combination of pregnancy and the low sodium intake led to the lowest adrenal sodium values.

No significant differences were observed in the potassium concentrations of the glands due to either pregnancy or dietary sodium.

A trend was noted toward increased water content of the adrenals during pregnancy. This was similar to observations on muscle reported for these animals (Kirksey and Pike, '62). This increase in water content was intensified in the low sodium pregnant group and this was true also in the case of muscle. The changes in water content of the adrenals and those previously reported in muscle were in-

TABLE 3

Average heart weights of nonpregnant and pregnant rats

Groups	Dietary Na	Total wet weight	Weight/100 gm body weight	
			Wet	Dry
	mEq/100 gm	*mg*	*mg*	*mg*
Nonpregnant	1.4	861 ± 13[1]	326 ± 3	74 ± 1
	6.3[2]	837 ± 14	324 ± 5	72 ± 1
	54.8	863 ± 22	336 ± 5	75 ± 1
Pregnant	1.4	794 ± 15	269 ± 7	62 ± 1
	6.3[2]	850 ± 6	230 ± 3	52 ± 1
	54.8	903 ± 20	247 ± 5	55 ± 1

[1] Standard error of the mean.
[2] Control group.

versely related to the changes in sodium concentration. Black ('60) has suggested that in a sodium deficiency the consequent increase in tissue water content is a result of the intracellular hydration which helps to maintain the plasma sodium concentration. Data for these animals reported previously indicated that plasma sodium concentration could not be maintained in pregnant animals on a low sodium intake and, in fact, decreased significantly despite the decrease in tissue sodium and the associated increased tissue hydration.

Average heart weights for nonpregnant and pregnant animals on the three levels of sodium intake are presented in table 3. Pregnancy did not affect the wet weight of the heart but the level of dietary sodium did produce a significant effect ($P = 0.01$) which was most evident in the pregnant groups, and was due to a significant interaction between pregnancy and sodium. The wet weights of hearts of pregnant animals increased with each increment of dietary sodium, the rats receiving the low sodium diet having the smallest hearts, and those receiving the high sodium diet during pregnancy having the largest hearts.

When heart weight was calculated on the basis of 100 gm of body weight, highly significant effects were observed due to pregnancy and to the level of sodium intake ($P = 0.001$). Pregnancy led to a decrease in relative heart weight due primarily to the greater increase in body weight compared with the increase in heart weight. The high intake of sodium caused a gain in the wet weights of hearts within both nonpregnant and pregnant groups. However, there was a significant interaction between pregnancy and dietary sodium ($P = 0.01$), which showed up in the smaller decreases in relative heart weight in pregnant animals fed low sodium diets compared with those receiving control or high levels of sodium. This was due, in part, to the smaller weight gains of these animals during pregnancy. Several workers including Meneely and Dahl ('61) and Tucker and his associates[5] observed that the relative weight of the heart of a rat and the level of blood pressure increased in proportion to the NaCl in the diet. Measurements in these animals, using an ankle cuff and a photoelectric tensometer, failed to show elevation of blood pressure in either the pregnant or nonpregnant animals. It is possible, however, that incipient hypertension, not detectable by the method of blood pressure determination employed, may have been associated with the increase in the relative wet and dry heart weight observed in these animals.

The sodium, potassium and water concentrations of hearts are presented in table 4. The sodium concentration of the heart decreased significantly during pregnancy ($P = 0.001$). This was associated with the decrease in both plasma and muscle sodium in pregnant animals reported previously. This change in cardiac sodium concentration was most pronounced in the pregnant animals receiving the low sodium diet. Each increment of sodium led to a highly significant in-

[5] Tucker, R. G., W. J. Darby and G. R. Meneely 1954 Effect of dietary NaCl on ultimate size, blood pressure and organ weight of rats. Federation Proc., 13: 480 (abstract).

TABLE 4

Average sodium, potassium and water content of hearts of nonpregnant and pregnant rats

Groups	Dietary Na	Sodium	Potassium	Water
	mEq/100 gm	*mEq/kg*	*mEq/kg*	%
Nonpregnant	1.4	39.27 ± 0.35[1]	68.35 ± 1.34	77.40 ± 0.18
	6.3[2]	41.57 ± 0.73	65.19 ± 0.93	77.67 ± 0.16
	54.8	42.43 ± 0.52	68.67 ± 0.92	77.50 ± 0.20
Pregnant	1.4	34.31 ± 1.00	63.85 ± 0.95	76.84 ± 0.20
	6.3[2]	40.10 ± 0.23	71.33 ± 0.61	77.21 ± 0.18
	54.8	41.52 ± 0.89	66.46 ± 0.93	77.71 ± 0.13

[1] Standard error of the mean.
[2] Control group.

crease ($P = 0.001$) in the sodium content of hearts in both nonpregnant and pregnant animals. This response was not dependent upon a change in plasma sodium since plasma sodium concentrations were significantly different only for the pregnant animals on the low sodium intake (Kirksey et al., '62). The amount of sodium in the diet had no significant effect on cardiac potassium content. The significant increase in cardiac sodium with high salt diets, without concomitant changes in potassium levels, lends support to Manery's ('54) theory that sodium and potassium movements in cells are controlled by separate mechanisms.

Cardiac potassium concentrations were not affected by pregnancy but a highly significant interaction ($P = 0.001$) between pregnancy and dietary sodium produced a decrease in the potassium content of hearts in pregnant rats fed the low sodium diet. This reduction in cardiac potassium was accompanied by elevated plasma potassium levels. It appears that pregnancy and dietary sodium restriction induced an increased secretion of aldosterone (Kumar et al., '59; Eisenstein, '57). The consequent increase in potassium excretion promoted by this hormone (Bartter, '56) may have led to a lowering of cardiac potassium levels. A corresponding increase in the cardiac sodium concentration due to the sodium-retaining action of aldosterone may have been prevented by the limited sodium available during dietary sodium restriction, or by the compensatory action of the "cell-sodium lowering factor" present in pregnancy, as postulated by Kumar et al. ('59).

No significant changes were noted in the water content of the heart due to pregnancy, sodium intake or to an interaction of the two, indicating that the increase in heart weight and sodium concentration in animals on the high sodium intake was not due to or associated with increased water content.

SUMMARY AND CONCLUSIONS

Adrenals and hearts from nonpregnant and pregnant rats fed three levels of dietary sodium were analyzed for sodium, potassium and water. The data correspond to and confirm data previously reported from this laboratory on plasma, muscle, bone and brain in these animals in that the combination of pregnancy and low sodium intake led to a significant reduction in the sodium concentration of maternal tissues and fluids. This suggests that there is an increased need for sodium during pregnancy. An increase in water content was associated with the decrease in sodium concentration of the adrenals as was true also for muscle. Water content of the heart, however, was not affected by the combination of low sodium and pregnancy; but cardiac potassium concentration was significantly decreased along with the decrease in sodium suggesting that the mechanisms for adjustment to reduced sodium concentration differ with different tissues.

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Digestibility of Threonine and Valine by Rats Fed Soybean Meal Rations[1]

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A recent publication from this laboratory (Borchers, '61) reported that rats fed a raw soybean meal ration required a supplement of methionine, threonine, and valine in order to maintain a growth rate comparable to those fed a ration containing heated soybean meal. In this connection, Booth et al. ('60) and Haines and Lyman ('61) suggested that the poor growth with raw soybeans may result from the loss of critical amounts of growth-limiting amino acids via fecal excretion of pancreatic enzymes. Previous publications have established the similarity in digestibility of sulfur (Johnson et al., '39) and methionine (Melnick et al., '46; Carroll et al., '53; Liener and Wada, '53) in rats fed heated and raw soybean. The methionine requirement of rats fed raw soybean meal rations (12% of protein) was observed by Borchers ('62) to be about 8% greater than in rats fed heated soybean. In view of the reported similarity in digestibility, this author suggested that the increased methionine need was caused by *in vivo* requirements of unknown nature.

The question then remains as to why supplementary amounts of threonine and valine are also needed to attain comparable growth rates with rations containing heated and raw soybean meal. This need could result from (1) decreased digestibility; (2) increased bacterial destruction in the intestinal tract or decreased intestinal synthesis; (3) increased loss via fecal excretion of pancreatic enzymes; or (4) increased metabolic requirements *in vivo* for threonine and valine. The following investigation of the digestibility of threonine and valine in rats fed soybean rations will provide information concerning the first and third of these possibilities. By similar studies with rations containing added antibiotics, information bearing on the second of these possibilities may be attained.

EXPERIMENTAL

Rations used in the following study were compounded as previously (Borchers et al., '57) and contained 25% of soybean meal (46.5% of protein, N × 6.25), 0.4% of supplementary DL-methionine,[2] minerals, vitamins, starch, and hydrogenated fat. Each ration was fed to a group of 8 weanling rats of the Holtzman strain for a 20-day period. The animals were housed individually in screen-bottom cages with food and water available ad libitum. Feces were collected on filter paper during the final 10 days of the feeding period, retrieved daily, and dried at 100°. Threonine and valine were determined in the soybean meal and the individual fecal collections by conventional titrimetric microbiological procedure following hydrochloric acid hydrolysis. The organism used was *Leuconostoc mesenteroides;* the media was that described for aspartic acid by Block and Weiss ('56) as modified from Barton-Wright ('46). Nitrogen was determined by macro-Kjeldahl procedure with a mercury catalyst.

RESULTS

The apparent digestibility of threonine and valine in rats fed a heated soybean meal ration was approximately 80%; in rats fed raw soybean, approximately 75%. In rations with added antibiotics, all values

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[2] DL-Methionine was graciously supplied by the Dow Chemical Company.

TABLE 1

Digestibility of threonine and valine by rats fed soybean meal rations

Soybean ration[1]	Gain		Digestibility[2]		
			Threonine	Valine	Nitrogen
	gm/day	*gain/food*	%	%	%
Heated	4.07 ± 0.18[3]	0.49 ± 0.01	81.3 ± 2.1	79.4 ± 1.9	78.7 ± 0.6
Raw	3.12 ± 0.22	0.43 ± 0.01	75.1 ± 2.6	73.2 ± 2.5	76.3 ± 0.9
$P <$	0.01	0.01			0.05
Heated + antibiotics[4]	4.01 ± 0.27	0.47 ± 0.01	76.6 ± 2.6	74.6 ± 1.9	77.7 ± 0.9
Raw + antibiotics	3.96 ± 0.14	0.47 ± 0.02	68.5 ± 3.4	67.3 ± 2.9	74.4 ± 1.0
$P <$					0.05

[1] The rations contained 25% of soybean meal, 46.5% of protein (N × 6.25). Each ration was fed to a group of 8 weanling rats.
[2] Digestibility values are apparent values not corrected for endogenous excretion.
[3] Standard error.
[4] Procaine penicillin plus streptomycin sulfate, each at a level of 0.1% of the ration.

were lowered 5 to 6 percentage points. The differences noted were not statistically significant. Apparent digestibilities of total nitrogen were generally similar with a smaller lowering with added antibiotics, the differences noted being significant at the 5% statistical confidence level. The results are presented in table 1.

DISCUSSION

The soybean rations plus antibiotics were included in the experimental design because of the possibility that the beneficial effects of antibiotics on the growth of rats fed raw soybean meal (Borchers et al., '57) might involve changes in digestibility. Both with and without added antibiotics, the differences in apparent digestibility of threonine, valine, and total nitrogen were of the same magnitude and statistical significance. Furthermore, the differences in digestibility noted in this study appeared to be of no consequence to the comparative growth performance of animals fed raw and heated soybean since the growth rates with the two rations were similar when supplemented with antibiotics.

The similarity in digestibility of threonine and valine in rats fed raw and heated soybean indicates quite clearly that the observed growth difference with the two dietary regimens does not result from fecal loss of critical amounts of these growth-limiting (for raw soybean) amino acids. This would eliminate the first and third of the possibilities suggested in the introduction, namely, decreased digestibility or increased loss via fecal excretion of pancreatic enzymes. Furthermore, the increase in pancreatic secretion observed with raw soybeans or the raw soybean trypsin inhibitor (Lyman and Lepkovsky, '57) does not result in loss of significant amounts of either total nitrogen or of threonine or valine. This does not exclude the possibility of involvement of recycling of the amino acids of the pancreatic enzyme proteins with a consequent growth reduction due to the increased work involved in synthesis of excessive amounts of pancreatic enzymes.

Of the other suggestions for the cause of the increased requirements for threonine and valine, namely, increased intestinal destruction or increased *in vivo* metabolic requirements, further work will be required to establish and elucidate the nature of the effects, if any, in this direction. The observed effect of antibiotics on rats fed raw soybeans (Borchers et al., '57) lends strong presumptive evidence for the involvement of intestinal bacteria in this problem. Whether this bacterial activity involves destruction of amino acids, decreased intestinal synthesis of amino

acids, liberation of heat-labile toxic factors from raw soybeans, or the formation of toxic compounds by bacterial fermentation remains obscure. The lack of significant effect of antibiotics on digestibility presents presumptive evidence that bacterial destruction is not involved, although this is by no means conclusive.

SUMMARY

The apparent digestibility of threonine and valine was determined in rats fed raw and heated soybean meal. The digestibility of both amino acids was about 80% in animals fed the heated soy and about 75% in those fed the raw soy; the differences were not statistically significant. The apparent digestibility of total nitrogen was found to be significantly less ($P < 0.05$), although the actual difference was small, with the raw soybean meal ration than with the heated meal. These differences persisted when a supplement of penicillin plus streptomycin was added, although the growth rates were similar in the latter case. The conclusion was drawn that the increased requirement for threonine and valine in rats fed raw soybean meal resulted from either increased intestinal destruction, decreased intestinal synthesis, or increased *in vivo* metabolic requirements of unknown nature.

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Dietary Proteins Correlated with Ribonuclease, Ribonucleic Acid, and Tissue Proteins[1]

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There is a need to study in more detail the impact of nutrition upon the metabolism of individual cells in the various tissues of the body. Previous studies demonstrate that this impact may differ from one tissue to another and that it is especially marked in liver cells (Allison and Fitzpatrick, '60). The nutritional regulation, for example, of protein and ribonucleic acid (RNA) metabolism in the liver cell has been studied extensively by Munro and his associates (Munro and Clark, '60). They found that the RNA content of the normal liver cell varied with the protein intake, a change that appeared to be associated with the RNA of the endoplasmic reticulum. This mechanism of control of protein synthesis was also influenced by the pattern of dietary amino acids. It has been reported further that RNA and the protein content of the liver increased and the activity of ribonuclease decreased with increasing nitrogen intakes (Zigman and Allison, '59; Allison et al., '61).

These and other studies suggest that the interrelationship between ribonuclease activity and RNA may play a role in controlling the rate of protein synthesis. The following experiments were planned, therefore, to lay a foundation for a series of studies on the interrelationships between ribonuclease activity, the RNA content of the liver, the magnitude of the protein reserves in various tissues of the body and the nutritive value of dietary protein.

METHODS

Male weanling Wistar rats were fed diets containing 9, 12, 18 or 25% of protein (Allison et al., '54). At each protein level, 10 rats were fed the diet ad libitum with daily food intakes recorded throughout the experiment. The proteins used in this study were egg albumin, casein,[2] cottonseed flour, and wheat gluten. After 28 days of feeding, the rats were killed and samples of liver, kidney, brain, muscle, and serum were taken for chemical analysis. During the experimental period, body weight measurements were performed weekly on each animal.

Serum proteins were separated by paper electrophoresis and stained with alcoholic bromphenol blue, whereas lipoproteins were stained with oil red O (Jencks et al., '55). Total serum proteins were determined by the Biuret method of Layne ('57), and ribonuclease activity at pH 7.4 was estimated by the technique of Brody ('57).

Liver, kidney, brain, and muscle tissues from each rat were homogenized in distilled water. This suspension was used to determine ribonuclease activity by the spectrophotometric method of Brody ('57), nucleic acid phosphorus concentration by a modified procedure of Schmidt and Thannhauser ('45), and protein by the Biuret technique. The homogenate was precipitated with trichloroacetic acid, washed with alcohol-ether mixtures, and dissolved in 1 N KOH. The resulting solution was used for the determination of ribonucleic acid and deoxyribonucleic acid-phosphorus by the method of Fiske and Subbarow ('25), and for the estimation of protein concentration by the Biuret technique which was standardized by the micro-Kjeldahl procedure of Pregl and Roth ('35).

Standard errors were determined for all values. A probability value where P is less

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[2] Labco, The Borden Company, New York.

than 0.01 was taken as a level of significance.

RESULTS

The results of these studies are summarized in figure 1. Weanling rats were fed egg protein, casein, cottonseed flour, or wheat gluten at different nitrogen intakes over a period of 28 days. The relationship between nitrogen intake and body weight gain is curvilinear but may be considered as linear at the lower nitrogen intakes. The slopes of these lines have been called the nitrogen growth indexes (Allison and Fitzpatrick, '60). The slopes of the lines drawn in figure 1 are approximately the same as those previously determined for the same proteins but in the previous studies many more points were used to define the course of each line. These slopes were egg protein, 33; casein, 23; cottonseed flour, 15; wheat gluten, 7. The nitrogen growth indexes have the same significance as the net protein ratio proposed by Bender and Doell ('57). This ratio is the weight loss of a negative control group added to the weight gain of the test group divided by the protein consumed by the test group. Calculations of this type overcome the variation in the protein-efficiency ratio with nitrogen intake (Barnes and Bosshardt, '46).

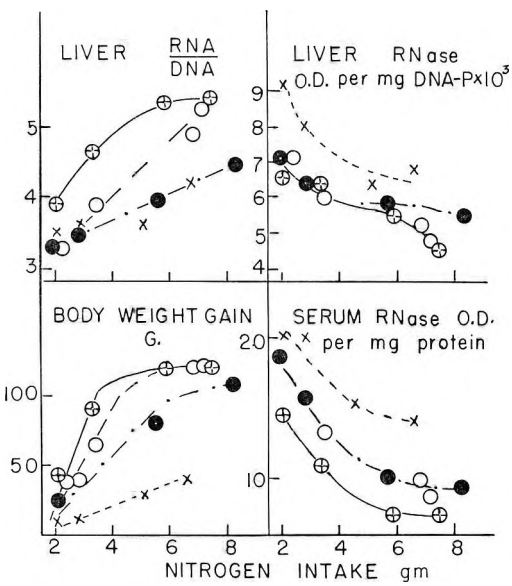

Fig. 1 The effects of nitrogen intake upon ribonucleic acid (RNA) and ribonuclease activity (RNase) relative to deoxyribonucleic acid (DNA) in the liver, serum ribonuclease activity and gain in body weight in rats fed a diet containing one of the following proteins: egg (circles with crosses), casein (open circles), cottonseed flour (closed circles) and wheat gluten (crosses).

Previous studies have demonstrated that liver cells of rats fed a protein-deficient diet rapidly lost a part of their protein and ribonucleic acid (Kosterlitz, '47; Campbell and Kosterlitz, '48, '52; Munro and Clark, '60). A reduction in cytoplasmic proteins could be correlated with a decrease in the ratio of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA). The liver RNA to DNA ratios were significantly greater in animals fed egg protein than in those fed wheat gluten or cottonseed flour. The ratio in animals fed casein was low at low nitrogen intakes, but approached values found in animals fed egg protein at high nitrogen intakes (fig. 1).

Munro and associates (Munro and Clark, '60) studied extensively this interrelationship between dietary protein intake and the RNA content of liver cells. They have emphasized the association of certain types of RNA with the endoplasmic reticulum which rises or falls with increase or decrease in nitrogen intake. A dynamic interchange between soluble RNA and the RNA of the endoplasmic reticulum and the ribosomes is considered. Their work suggests that the formation of RNA may not be a direct function of nitrogen intake but that there is some mechanism of control associated with the quantity of amino acids in the various metabolic pools. It is possible that the RNA not involved in amino acid synthesis into tissue protein may be hydrolyzed by ribonuclease, an enzyme that is resistant to depletion. Previous studies have demonstrated that the relative increase in ribonuclease activity in tumor tissue resulted in a reduction in RNA and a cessation in growth of the tumor. (Zigman and Allison, '59; Allison et al., '61.) The shape of the RNA/DNA curve (fig. 1), for animals fed egg protein, could be interpreted to mean that a point of saturation for RNA in protein synthesis was reached at the higher intakes. In other words, a high intake of the pattern of amino acids provided by egg protein may protect RNA from catabolic processes. Such a suggestion, of

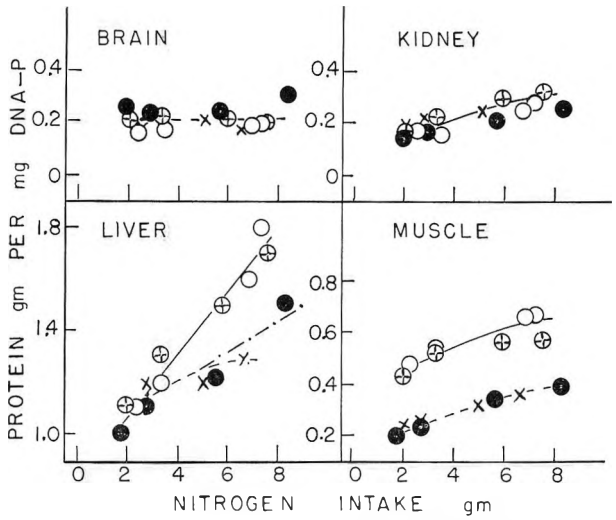

Fig. 2 The effects of nitrogen intake upon tissue proteins relative to DNA in rats fed a diet containing one of the proteins recorded under figure 1.

course, can be evaluated only by further study. The data presented in figure 1 demonstrate that the RNA/DNA ratio is influenced by the pattern of amino acids; the more deficient the pattern the less RNA in the cells. With high nitrogen intakes of casein the ratio became equivalent to those observed for animals fed egg protein which would be expected if the pattern of amino acids in casein was not too dissimilar from the pattern in egg protein. Ratios to DNA were used in figures 1 and 2 so that the variables could be approximately correlated with the nuclear content of the tissues. Such a correlation eliminates, at least in part, changes associated with alterations in lipid or water content of the tissues.

The ribonuclease activity in the liver relative to DNA was not reduced by low nitrogen intakes but tended to increase with decreased dietary nitrogen and to be higher in rats fed the protein with lowest nutritive value. The ribonuclease activity relative to serum protein also decreased with increasing nitrogen intake, and was significantly higher in animals fed wheat gluten than in those fed the other proteins. This ratio in the serum was significantly lowest in animals fed egg protein. Thus, the RNA was highest and the relative ribonuclease activity lowest in animals fed egg, with a rough correlation between these variables and the nutritive value of the dietary proteins as determined by the nitrogen growth indexes.

The nutritive value of dietary protein with respect to protein synthesis in individual tissues may vary from tissue to tissue and need not correlate with the overall gain in body weight. The ratios between protein and DNA in different tissues are plotted in figure 2. The protein in each liver cell may be considered as increasing with nitrogen intake, the greatest increases being observed in animals fed egg or casein nitrogen. The liver protein was higher in animals fed egg or casein than in those fed cottonseed flour and wheat gluten. This separation between egg or casein and cottonseed flour or wheat gluten was as marked, even more so, at low nitrogen intakes in the muscle cells. The total protein in the kidney with respect to DNA increased with nitrogen intake but there were no significant differences in this ratio between rats fed egg, casein, cottonseed, or wheat gluten. Similarly, the overall nutritive value of the dietary protein did not affect the total protein in the brain with respect to DNA and this ratio was independent of nitrogen intake. Previous studies involving the resistance of various tissues to depletion of protein in the rat demonstrated that the brain was most resistant, followed by the kidney, then the

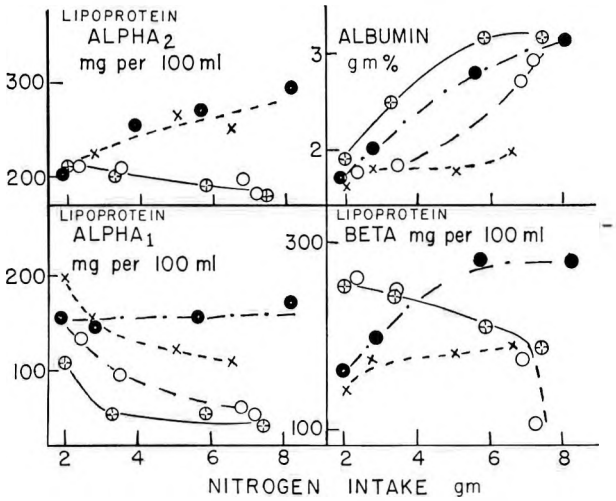

Fig. 3 The effect of nitrogen intake upon lipids migrating electrophoretically with alpha$_1$, alpha$_2$, or beta globulins in rats fed a diet containing one of the proteins recorded under figure 1. The effect of nitrogen intake upon serum albumin is illustrated in the upper right portion.

muscle and the liver (Wainio et al., '59; Allison and Fitzpatrick, '60). A great need exists to determine the physiological significance of variations in cellular proteins, revealed by these data, especially as they may be correlated with the concept of "protein reserves."

The different effects of casein and egg protein compared with those of cottonseed flour and wheat gluten upon the biochemistry of the body are illustrated further in figure 3. The lipoproteins, alpha$_1$, alpha$_2$, and beta are recorded in this figure. In general, the quantity of lipid migrating electrophoretically with the alpha and beta proteins decreased with increasing nitrogen intake in animals fed egg or casein especially at the higher nitrogen intakes. The beta lipid fraction, on the other hand, increased markedly with increased intake of cottonseed flour. These results are in agreement with those reported by Spoerlein,[3] and recall the data on the effect of repletion with casein or wheat gluten of protein-depleted dogs. Depletion of the dogs resulted in a marked lipemia which was corrected by repletion with casein but not with wheat gluten (Allison and Wannemacher, '61).

The serum albumin concentrations obtained in this experiment are recorded in the upper right portion of figure 3. The relatively reduced albumin concentrations in rats fed casein at lower nitrogen intakes has been associated with a sulphur amino acid deficiency. Supplementation with methionine increased the albumin concentration in both dogs and rats fed casein (Allison and Wannemacher, '57).

These studies support the suggestion that feeding a dietary protein with a nutritive value below some critical figure will result in an imbalance in growth of certain tissues (Allison and Fitzpatrick, '60). It should be emphasized, however, that diets usually contain mixed proteins so that the nutritive value obtained for a single protein cannot be used to evaluate that protein in a mixed diet. The cottonseed flour, for example, used in these studies had a nitrogen growth index below casein. Cottonseed flour, however, combined with maize, sorghum and yeast resulted in a mixture with a nitrogen growth index close to casein.[4]

SUMMARY

The nitrogen growth indexes for egg, casein, cottonseed flour and wheat gluten

[3] Spoerlein, M. T. 1959 The effect of dietary proteins on the serum lipids of normal and tumor-bearing rats. Ph.D. Thesis, Rutgers University, New Brunswick, New Jersey.

[4] Scrimshaw, N. S., and R. Bressani 1961 Vegetable protein mixtures for human consumption. Federation Proc., 20: 80 (abstract).

were determined for weanling rats. The ratio between RNA and DNA in the liver was highest in rats fed egg, lowest in those fed cottonseed flour or wheat gluten and in between in those fed casein. The ribonuclease activity in the liver was not reduced by low nitrogen intakes but tended to increase with decreased dietary nitrogen and to be higher in rats fed wheat gluten. Serum ribonuclease activity relative to serum protein was highest in animals fed wheat gluten, lowest in animals fed egg, and in between in animals fed casein or cottonseed flour.

Total protein with respect to DNA increased in the liver as nitrogen intake was raised and the greatest increases were observed in rats fed egg and casein proteins. A similar increase was found in the muscle where the ratios were higher in animals fed egg or casein than in those fed wheat gluten or cottonseed flour. Protein relative to DNA also increased in the kidney with nitrogen intake but no difference was observed between animals fed the various types of protein. The protein/DNA ratio in the brain was independent of nitrogen intake or the nutritive value of the dietary protein.

The lipids migrating electrophoretically with the plasma globulins were, in general, higher in animals fed cottonseed flour or wheat gluten than in animals fed egg or casein.

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Iron Content of Liver in Relation to Age[1]

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The level of iron in the liver reflects the amount of iron stored in the body in many conditions. This has been noted in rats in various experiments related to iron metabolism and absorption (Kinney et al., '55; Klavins et al., '62). However, it has been observed that when rats suffered from anemia, there was no correlation between the values for iron in the liver and total body iron (Klavins et al., '62). During the course of various experiments the question of the influence of age upon the relationship between liver iron values and hemoglobin was raised. Since it is important to know the physiological values for liver iron and hemoglobin in many types of studies, the present experiment was designed to investigate the liver iron changes in rats in relation to age and to correlate these values with growth of the animal, liver weight and hemoglobin values.

MATERIALS AND METHODS

Seventy-five male albino rats of the Sprague-Dawley strain with an average weight of 85 gm were used. They were 4 weeks old at the beginning of the experiment. The animals were housed individually, weighed twice a week, and fed a standard laboratory diet[2] ad libitum. An acceptable, widely used commercial diet rather than a synthetic diet was selected since it was felt that the observations would reflect more faithfully the iron content of livers in stock laboratory animals in relation to age. Eight animals were killed at the beginning of the experiment and every 4 weeks thereafter. After 32 weeks, when the rats were 36 weeks old, the remaining 11 animals were killed. The animals were killed with ether and autopsied. The liver was removed, weighed and analyzed for iron without prior perfusion. The analysis was performed by the method of Kitzes et al. ('44) after sulphuric, nitric and perchloric acid digestion. Hemoglobin values were determined by the cyanmethemoglobin method and read on a Klett colorimeter. Data were analyzed using Student's t test.

RESULTS AND DISCUSSION

The liver iron values are presented in table 1. The most striking changes occurred during the first 4 weeks of the experiment when the animals were 4 to 8 weeks old. A rapid increase in total liver iron occurred during this period ($P < 0.01$). This was followed by a more gradual but still significant increase up to 20 weeks of the experiment when the animals were 24 weeks old.

No statistically significant difference was found between the groups killed at ages 16 and 20 weeks ($P > 0.1$), but a statistically significant difference was noted between 16 and 24 weeks and 20 and 24 weeks of age ($P < 0.05$). No statistically significant variation in total liver iron was noted in any of the subsequent groups when compared with the 24-week-old rats. Concomitantly, the concentration of the iron in the liver presented the same pattern.

When the liver iron was calculated in terms of milligrams per 100 grams of liver a statistically significant gain appeared in the 8-week-old rats when compared with the 4-week-old rats ($P < 0.01$) and again between 20 and 24 weeks of age ($P < 0.05$). There were no significant differences between the other groups. From this it can be seen that increase in liver iron is not due to increase in liver weight alone, but there is an increased concentration of iron during the early life of the rat.

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[1] This work supported by grant number A-4839 from U.S. Public Health Service.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis; contained 329 ppm of iron and 25% of protein according to the manufacturer's analysis.

TABLE 1

Changes in liver iron values and hemoglobin values with age

Age	No. of animals	Total liver iron	Liver iron	Average liver weight	Avg body wt	Total liver iron/ 100 gm body weight	Hemoglobin
weeks		*mg*	*mg/100 gm liver*	*gm*	*gm*	*mg*	*gm/100 ml blood*
4	8	0.247	6.0	3.99	85	0.291	9.22
8	8	0.932	10.3	9.71	213	0.438	11.49
12	8	1.362	11.1	12.44	316	0.431	13.90
16	8	1.760	11.2	15.65	373	0.472	15.29
20	7[1]	1.739	11.5	15.15	406	0.428	14.52
24	7[1]	2.371	14.6	16.14	442	0.536	14.72
28	8	2.333	14.4	16.32	455	0.514	14.77
32	8	2.189	12.6	17.33	430	0.509	13.31
36	11	2.083	12.6	16.53	474	0.439	14.85

[1] The value for one animal in each of these groups was more than two standard deviations from the mean value and these two animals were excluded.

There was a statistically significant increase in liver weight between the 4- and 8-week-old rats ($P < 0.01$) 12- and 16-week ($P < 0.05$) and 20- and 24-week-old animals ($P < 0.05$). No significant differences were seen in other groups.

The animals grew steadily but the fastest growth appeared during the early part of the experiment. The most rapid period of growth occurred between 4 and 8 weeks, and 8 and 12 weeks of age. When the total liver iron was calculated per 100 gm of body weight a statistically significant increase was noted during the first period and again between 16 and 20 weeks of the experiment; i.e., when the animals were 20 to 24 weeks old. This indicates that at these intervals there was an increase in liver iron beyond that expected from growth alone of the animal.

At the beginning of the experiment the rats had low hemoglobin values which increased notably during the first 4 months of the experiment. A statistically significant increase in hemoglobin was found in the animals at 4 and 8 weeks of age ($P < 0.01$) 8 to 12 weeks ($P < 0.01$) and 12 and 16 weeks of age ($P < 0.05$). No statistically significant differences were seen in the other groups.

It is generally known that young animals have low hemoglobin values. Whether this is physiologic or whether it represents a deficiency anemia is not clear. Von Bidder and Undritz ('48) considered the anemia in young animals to be alimentary and not physiological.

Von Bidder and Undritz ('48) found low liver iron values in young rats. At 40 days of age, the average was 6.64 mg/100 gm of wet liver, and at 312 days, the average was 14.34 mg/100 gm of wet liver. These authors did not attempt to correlate their findings with the liver weights of the animals or with their hemoglobin values. Similarly, Lintzel and Radeff ('30) found low liver iron values in young animals. In two male rats weighing 134 and 140 gm, the total liver iron was 0.57 and 0.52 mg, respectively, and two older male rats weighing 207 and 216 gm, the total liver iron values were 1.29 and 1.46 mg, respectively. It is not possible to compare these results with ours in detail since the condition of their experiments, the experimental design, and method of iron determination were different.

In the present study the low liver iron values at the beginning of the experiment could be explained on the basis of the associated anemia, so that the liver iron stores were constantly being depleted by rapid hemoglobin synthesis. As the animals were fed the stock diet which contained 329 ppm of iron, the iron in the diet was sufficient to compensate for the needs of hemoglobin synthesis as well as for the repletion of iron stores. In the 16- to 20-week interval, despite the constant hemoglobin values, the liver iron still in-

creased significantly. From these experiments it can be concluded that there is a continuous deposition of liver iron as the animals grow older, until they attain a weight of 450 gm and are approximately 24 weeks old.

SUMMARY

Sprague-Dawley male albino rats were fed a stock diet containing 329 ppm of iron for 32 weeks. Groups of animals were killed at the beginning of the experiment and at 4-week intervals thereafter. It was found that at the beginning of the experiment the animals had low liver iron and low hemoglobin values which increased with time and then became stabilized. The increase in liver iron continued beyond the time that hemoglobin values became constant. In general the liver iron concentrations paralleled the total liver iron.

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Influence of Protein Depletion on Vitamin A and Carotene Utilization by Vitamin A-deficient Sheep[1,2,3]

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There is considerable evidence in the literature which indicates that vitamin A in both blood and liver is linked to protein. As early as 1914, Palmer and Ekles ('14) showed that blood plasma carotenoids could not be extracted until the protein had been denatured with ethanol to release the carotene. Erwin et al. ('59) reported that vitamin A in the blood serum of calves was associated primarily with albumin, while Garbers et al. ('60), administering isotopically labeled vitamin A to rats, found the vitamin A associated with the globulin fractions. Although there is some confusion as to the specific protein fraction with which vitamin A is associated, it is accepted that vitamin A occurs in the blood linked to protein.

A recent study by Friend et al. ('61) of the relationship between protein deficiency and the transport of vitamin A in the blood showed a significant correlation ($r = 0.76$) between the concentration of vitamin A and albumin in the serum of young pigs. Pigs fed protein-deficient diets stored approximately 20% of a large single dose of vitamin A in their livers, whereas the animals receiving adequate dietary protein stored 80%. This research suggested that dietary protein deficiency limits the production of the serum protein fraction carrying vitamin A and that the insufficient protein carrier will both inhibit the mobilization of stored hepatic vitamin A and the absorption of vitamin A through the intestinal wall.

Under range conditions, periods of low-protein intake are usually coincident with periods of low-carotene content in the forage. The objectives of this study were to determine the influence of dietary protein level upon total serum protein and serum protein fractions, conversion of carotene to vitamin A, and hepatic vitamin A storage.

MATERIALS AND METHODS

Seventeen mature ewes and wethers of mixed breeding, deficient in vitamin A, were used in this study. Nine of the animals received a protein-deficient ration (5.9% crude protein [CP]), and the remaining 8 sheep were fed a ration adequate in protein (10.4%). Analysis revealed no measurable carotene in either of the two rations fed. Allotment of treatments was made on the basis of liver biopsy vitamin A values determined two months previously. The composition of the two essentially carotene-free rations is presented in table 1.

TABLE 1
Composition of the experimental rations

	10.4% Crude protein ration	5.9% Crude protein ration
	lb	*lb*
Cottonseed hulls	715	835
Milo	150	150
Cottonseed meal	110	—
Urea	10	—
Salt	10	10
Bonemeal	5	5
Total	1,000	1,000

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[3] From a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

Phase 1. The experiment was conducted in two phases. The first period of 8 weeks (October 11, 1960 to December 6, 1960) consisted of a preliminary protein depletion phase. Symptoms of protein deficiency were verified by electrophoretic fractionation of blood serum and the measurement of total serum protein, hematocrit and weight loss. At the end of the first phase the sheep were bled by venous puncture and liver samples were taken by the aspiration biopsy technique.

Phase 2. During the second period of 4 weeks (December 6, 1960 to January 3, 1961) both the protein-deficient and protein-adequate groups received daily intraruminal injections of either vitamin A acetate or carotene. Within each of the two protein levels the sheep were divided into control, vitamin A and carotene treatments (table 2).

Vitamin A was injected intraruminally at the rate of 0.1 mg of vitamin A acetate/kg of body weight daily and carotene was injected intraruminally at the rate of 0.2 mg of β-carotene /kg of body weight daily. The injections were based on weights obtained on December 6, 1960. The vitamin A and carotene solutions were freshly prepared each week in concentrations of 1 mg/ml, according to the method of Bieri ('51), and stored at 5°C. At completion of this phase all sheep were bled by venous puncture and slaughtered to obtain data on the liver weights.

The blood samples obtained during the course of the experiment were analyzed for total serum protein, plasma vitamin A, hematocrit and electrophoretic separation of serum protein fractions. Total serum protein was determined with the Bausch and Lomb refractometer Model ABBE-3L, according to the procedure outlined by Sunderman ('44), and plasma vitamin A by the method of Kimble ('39). Per cent blood cell volume was obtained by the micro-hematocrit technique and was read on an International rotary micro-capillary reader. Serum proteins were fractionated using the Spinco Model R paper electrophoresis cell, Spinco Duostat and Model RB analytrol. The liver samples taken at the end of each phase were analyzed for vitamin A and carotene concentration by the method of Gallup and Hoefer ('46).

In addition to the above, sheep were weighed initially and at the end of each phase of the experiment. The rations were also analyzed for crude protein and carotene content as described by the AOAC ('55).

The experiment was set up as a single replication of a completely randomized design and the data were subjected to an analysis of variance using the method of unweighted means as outlined by Snedecor ('56).

RESULTS AND DISCUSSION

Phase 1 (October 11 to December 6, 1960). When the experiment was initiated the only symptoms of vitamin A deficiency observed in the sheep were general excitability and occasional cases of muscular incoordination. These observations are in agreement with Klosterman et al. ('49) who found no clinical symptoms in vitamin A-deficient lambs, except those mentioned above.

Table 3 presents the treatment effects noted at the beginning and end of the protein depletion phase. The group fed the 10.4% CP ration had initial plasma vitamin A levels averaging 14.1 μg/100 ml, whereas the sheep receiving the 5.9% ration averaged 12.6 μg/100 ml of plasma vitamin A. After the 8-week protein depletion period, the sheep fed the 5.9% CP

TABLE 2

Design of experiment with number of animals per treatment

	10.4% Crude protein ration			5.9% Crude protein ration		
Phase 1[1]	8			9		
	10.4% Crude protein ration			5.9% Crude protein ration		
	Control	Carotene	Vitamin A	Control	Carotene	Vitamin A
Phase 2[2]	2	3	3	3	3	3

[1] (October 11, 1960 to December 6, 1960).
[2] (December 6, 1960 to January 3, 1961).

TABLE 3

Influence of dietary crude protein level on blood plasma vitamin A, total serum protein, serum albumin/globulin ratio and hepatic vitamin A storage — phase 1

Component	10.4% Crude protein ration		5.9% Crude protein ration	
	Initial (10/11/60)	End first phase (12/6/60)	Initial (10/11/60)	End first phase (12/6/60)
Blood plasma vitamin A, μg/100 ml	14.1 ±2.3[1]	10.9 ±0.78	12.6 ±2.1	4.3 ±0.73
Total serum protein, gm/100 ml	6.58±0.20	6.87±0.24	6.63±0.18	6.53±0.23
Serum albumin, gm/100 ml	3.55±0.15	3.93±0.13	3.31±0.14	3.22±0.12
Albumin/globulin ratio	1.25±0.12	1.39±0.08	1.14±0.11	1.01±0.06
Hepatic vitamin A, μg/gm	7.0 ±3.1[2]	3.8 ±0.70	6.5 ±2.7[2]	5.2 ±0.66
Change in hepatic vitamin A, %		−45.7		−20.0

[1] Standard error of the mean, see table 2 for number of animals/treatment.
[2] Biopsied 8/2/60.

ration showed a highly significant depression in plasma vitamin A ($P < 0.01$).

Compared with initial values, the variation in plasma vitamin A levels within each protein level was less at the end of the depletion phase. As the sheep became further depleted in vitamin A it would be expected that vitamin A reserves would be used more efficiently. The sheep with higher initial plasma vitamin A levels showed a relatively greater loss in circulating vitamin A during the 8-week protein depletion phase than those sheep with low initial plasma vitamin A levels. This finding was demonstrated by the smaller standard errors in the plasma vitamin A determinations at the end of the protein depletion period, compared with the initial values.

Analysis of the liver biopsy samples taken at the end of this phase revealed that the protein-adequate sheep had a slightly lower average hepatic vitamin A concentration (3.8 μg/gm of fresh liver) than the protein-deficient animals (5.2 μg/gm of fresh liver).

Since both groups of animals were fed the vitamin A- and carotene-deficient diets during this phase of study, the primary source of plasma vitamin A was the liver stores. The loss of only 20.0% of hepatic vitamin A concentration during the 8-week period for the protein-deficient group as compared with a 45.7% loss ($P < 0.01$) for the sheep receiving adequate dietary protein suggests the possible failure of mobilization or transport mechanisms, or both, for vitamin A. The slight increase in body weight and presumably heavier livers for the sheep fed the adequate protein diet, compared with an average 23-pound weight loss for the sheep fed the protein-deficient diet undoubtedly also contributed to the observed differences.

Serum proteins have been implicated in other studies as the transporting mechanism for vitamin A (Arroyave et al., '61; Friend et al., '61). That a reduced level of serum proteins could have been responsible for the apparent failure of the vitamin A transporting mechanism in this study is illustrated by the changes in total serum protein and serum protein fractions that resulted from the levels of protein fed. Total serum protein for the 10.4% CP and 5.9% CP groups at the end of the first phase was 6.87 gm/100 ml and 6.53 gm/100 ml, respectively. This decrease in total serum protein in the protein-depleted sheep was not statistically significant, however, serum albumin expressed in grams/100 ml was significantly lower ($P < 0.05$) in the protein-deficient sheep at the end of the 8-week depletion period. This pattern of alteration in the serum protein fractions supports the earlier research of Klosterman et al. ('50), with pregnant ewes, and Peo et al. ('57), working with protein-depleted baby pigs.

The effects of protein depletion were also noted in the albumin: globulin (A:G) ratio at the end of the first phase, with the protein-depleted sheep showing a significantly lower A:G ratio ($P < 0.05$) than

the sheep receiving adequate protein in the ration. Peo et al. ('57), in their research with protein-depleted pigs, found that the A:G ratio was one of the most promising indices of protein deficiency. Using both protein depletion and repletion, it was found that the A:G ratio was reduced by protein depletion and returned to the initial level upon protein repletion.

Hematocrits taken at the end of this phase were significantly lower ($P < 0.05$) for the protein-depleted sheep (34.4%) than for the sheep fed the adequate protein ration (41.3%). However, it was not possible to determine whether this lower value was due solely to protein deficiency since no initial values were obtained.

At the beginning of the experiment body weights of the sheep fed adequate protein and those fed the protein-deficient ration averaged 147 and 148 pounds, respectively. At the end of the protein-depletion period the protein-deficient sheep averaged 125 pounds, whereas the average weight of the protein-adequate animals increased to 156 pounds. The 31-pound/head difference in average body weight between the two protein levels was highly significant ($P < 0.01$).

Phase 2 (December 6, 1960 to January 3, 1961). During this period sheep fed both the 5.9% CP and 10.4% CP rations received daily intraruminal injections of either vitamin A acetate or β-carotene. Within each of the two protein levels, approximately one-third of the sheep served as controls, one-third received 0.1 mg of vitamin A acetate/kg of body weight, and one-third received 0.2 mg β-carotene/kg of body weight (table 2).

The treatment effects noted during the 4-week vitamin A and carotene supplementation period are presented in table 4.

Plasma vitamin A levels of all sheep receiving intraruminal injections of either vitamin A or carotene were significantly higher than those of the controls ($P < 0.01$) regardless of the level of protein fed. The plasma vitamin A levels of the controls fed adequate protein decreased from 10.5 to 4.9 μg/100 ml, whereas the protein-deficient controls maintained a relatively constant level (3.0 to 3.8 μg/100 ml). This indicated that the livers of the sheep fed the adequate protein ration were

TABLE 4

Influence of dietary crude protein level and intraruminal injections of carotene or vitamin A on blood plasma vitamin A, total serum protein, serum albumin, albumin/globulin ratio and hepatic vitamin A storage — phase 2

Component	Date	10.4% Crude protein ration				5.9% Crude protein ration			
		Control	Carotene	Vitamin A	Avg	Control	Carotene	Vitamin A	Avg
Blood plasma vitamin A, μg/100 ml	12/6	10.5 ±2.0[1]	9.0 ±1.6	14.0 ±1.6	10.9	3.0 ±1.6	2.6 ±1.6	7.2 ±1.6	4.3
	1/3	4.9 ±5.2	8.6 ±4.3	68.3 ±4.3	30.1	3.8 ±4.3	6.6 ±4.3	31.4 ±4.3	13.9
Total serum protein, gm/100 ml	12/6	6.25±0.56	6.79±0.46	7.35±0.46	6.87	6.30±0.46	6.84±0.46	6.44±0.46	6.53
	1/3	5.92±0.53	6.67±0.42	6.91±0.42	6.57	5.94±0.42	6.42±0.42	5.89±0.42	6.08
Serum albumin, gm/100 ml	12/6	4.04±0.25	4.07±0.21	3.72±0.21	3.93	2.94±0.21	3.31±0.21	3.40±0.21	3.22
	1/3	3.40±0.19	3.99±0.15	3.74±0.15	3.75	2.98±0.15	2.57±0.15	2.35±0.15	2.63
Albumin/globulin ratio	12/6	1.83±0.17	1.21±0.14	1.29±0.14	1.39	0.92±0.14	1.00±0.14	1.12±0.14	1.01
	1/3	1.38±0.57	1.53±0.46	1.26±0.46	1.39	1.01±0.46	0.68±0.46	0.67±0.46	0.79
Hepatic vitamin A, μg/gm	12/6	3.7 ±1.4	4.0 ±1.1	3.5 ±1.1	3.8	2.8 ±2.0[2]	4.7 ±1.1	6.5 ±1.1	5.2
	1/3	2.3 ±8.5	7.1 ±6.9	74.7 ±6.9	31.3	2.7 ±6.9	8.0 ±6.9	71.6 ±6.9	27.4

[1] Standard error of the mean; see table 2 for number of animals/treatment.

[2] One observation.

essentially depleted. Vitamin A storage data (table 5) showed approximately 1 mg of vitamin A remaining in both treatments.

Injections of vitamin A increased the plasma vitamin A level of the protein-adequate sheep 54.3 μg/100 ml and the protein-deficient sheep 24.2 μg/100 ml. Biopsy data indicated essentially the same vitamin A storage per gram of tissue (74.7 vs. 71.6 μg) for the animals fed the 10.4 and 5.9% CP rations, respectively. However, the total liver storage data (table 5) revealed that the group fed adequate protein had stored approximately twice as much vitamin A as the protein-deficient sheep (66.6 vs. 32.1 mg of vitamin A) due to the difference in liver weights. There were no significant differences in liver weight between the two protein levels when liver weight was expressed as a percentage of body weight, due to marked individual variation between animals within the same treatment.

Total serum protein continued to be depressed in the protein-deficient group but an average difference of 0.5 gm/100 ml between the two protein groups was not statistically significant. No treatment effects on total serum protein were observed as a result of the vitamin A or carotene injections.

The serum albumin of the protein-depleted animals continued at a significantly lower level ($P < 0.01$) than that of the sheep fed the adequate protein rations. The A:G ratio also continued to be significantly depressed ($P < 0.01$) in the protein-depleted animals. No treatment effects as a result of the vitamin A or carotene injections were noted with either serum albumin or the A:G ratio.

Hematocrits taken at the end of the 4-week supplementation period were lower in the protein-deficient sheep (32.7%) than in the sheep fed the adequate protein ration (38.7%); however, the difference was not found to be statistically significant.

Hepatic vitamin A storage of all sheep receiving intraruminal injections of vitamin A acetate or β-carotene, measured in micrograms per gram of fresh liver, was significantly higher than the controls ($P < 0.01$), regardless of the level of protein in the ration. The sheep receiving intraruminal injections of vitamin A acetate stored approximately 10 times more hepatic vitamin A than the animals receiving injections of β-carotene, regardless of the protein level. This difference was highly significant ($P < 0.01$). It was found, however, that evaluating the vitamin A status of an animal in terms of storage per unit of liver tissue can be misleading because of total liver weight changes resulting from treatment influence.

Table 5 shows that the liver weights of the sheep receiving adequate protein were significantly heavier than those of the protein-deficient sheep ($P < 0.05$). The control animals had significantly lighter livers than the sheep receiving injections of vitamin A acetate or β-carotene, regardless of the level of protein ($P < 0.05$). In addition, the sheep receiving injections of vitamin A acetate had significantly heavier livers than those animals receiving injections of

TABLE 5

Influence of dietary crude protein level and intraruminal injections of carotene or vitamin A on liver weight and hepatic vitamin A storage

Component	10.4% Crude protein ration				5.9% Crude protein ration			
	Control	Carotene	Vitamin A	Avg	Control	Carotene	Vitamin A	Avg
Liver weight, gm	466±73[1]	525±60	892±60	648	303±60	424±60	449±60	392
Hepatic vitamin A, μg/gm	2.3±8.5	7.1±6.9	74.7±6.9	31.3	2.7±6.9	8.0±6.9	71.6±6.9	27.4
Total hepatic vitamin A, mg	1.1±5.9	3.7±4.8	66.6±4.8	26.7	0.8±4.8	3.4±4.8	32.1±4.8	11.9
Total dosage, mg		372	210			323	156	
Percentage of total dosage retained as hepatic vitamin A		0.7	31.2			0.8	20.1	

[1] Standard error of the mean; see table 2 for number of animals/treatment.

β-carotene, regardless of the protein level ($P < 0.01$). The significant influence of carotene and vitamin A administration on liver weight may be a reflection of increased ration consumption on both protein levels since all animals were fed on an ad libitum basis. There was essentially no influence of the protein level of the diet on total hepatic storage of vitamin A for the control or carotene injection treatments.

The intraruminal injections of vitamin A acetate (0.1 mg/kg of body weight), measured by total hepatic vitamin A, were approximately 9 times more effective than the intraruminal injections of β-carotene (0.2 mg/kg of body weight) in the protein-deficient sheep and more than 18 times as effective in sheep receiving adequate protein ($P < 0.01$). This effect is due to the heavier livers of the vitamin A-injected sheep fed the 10.4% CP ration, since in terms of vitamin A storage per unit of liver tissue, there was no difference between the two protein levels.

The level of dietary protein fed had no appreciable effect on the efficiency of conversion of carotene to vitamin A. The carotene injections (0.2 mg/kg of body weight), which were given at a rate 8 times the minimum daily requirement for sheep recommended by the National Research Council ('57), were not utilized efficiently enough to maintain the initial levels of plasma and hepatic vitamin A. This suggests that vitamin A-deficient sheep cannot convert crystalline β-carotene to vitamin A at a rate sufficient to meet the physiological needs of the animal.

SUMMARY

Seventeen vitamin A-depleted sheep were fed either a protein-deficient (5.9% crude protein) or a protein-adequate (10.4% crude protein) ration. At the end of an 8-week protein - depletion period, the 9 sheep in the protein-deficient group and the 8 sheep receiving adequate dietary protein were biopsied for liver samples and allotted to the following treatments: control, daily intraruminal injections of 0.1 mg vitamin A acetate/kg of body weight and daily intraruminal injections of 0.2 mg of β-carotene/kg of body weight. All sheep were killed at the completion of a 4-week injection period to obtain liver weights.

Significant decreases in serum albumin, A:G ratio, plasma vitamin A and body weight were noted in the sheep fed the protein-deficient ration at the end of the protein depletion period and also at the termination of the 4-week injection period. When vitamin A acetate was injected intraruminally and storage was expressed in terms of total liver storage, the sheep fed 10.4% crude protein ration stored more than twice as much vitamin A (66.6 mg) as those fed the 5.9% crude protein ration (32.1 mg).

Level of protein was without appreciable influence on carotene conversion to vitamin A. The intraruminal vitamin A injections (0.1 mg/kg of body weight), measured by total hepatic vitamin A, were approximately 9 times more effective than the intraruminal injections of carotene (0.2 mg/kg of body weight) in the protein-deficient group and more than 18 times as effective in the sheep receiving adequate protein. The average plasma vitamin A levels of the sheep receiving the 10.4% crude protein ration increased more than 113% above the initial levels during the 4-week injection period and increased only 10% in similarly treated sheep fed the 5.9% crude protein ration.

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Tissue Uptake and Urinary Excretion of Radioactive Cobamide Analogues and Inorganic Cobalt in Growing Female Rabbits[1,2]

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Previous reports have demonstrated that kidneys from starved female rabbits accumulate considerably more native vitamin B_{12} (Rosenthal and Cravitz, '58) or injected Co^{60}-cyanocobalamin (Rosenthal, '59, '61a) than fed animals. Nephrotoxic agents tended to eliminate the increased accumulation of the vitamin in the kidneys of starving rabbits (Rosenthal, '61b), whereas thiouracil feeding increased the accumulation in fed rabbits (Rosenthal and O'Brien, '62). Chosy et al. ('62), showed that hydroxocobalamin is more tightly bound to serum and liver proteins than cyanocobalamin and Samson et al.[3] observed that hydroxocobalamin and coenzyme B_{12} were retained better than cyanocobalamin in human subjects following intramuscular injection. Glass et al.[4] also found that hydroxocobalamin is superior to cyanocobalamin in maintaining vitamin B_{12} serum levels. It seemed pertinent therefore to compare the accumulation of these cobamide analogues and inorganic cobalt in tissues of starved female rabbits.

MATERIAL AND METHODS

White female rabbits, obtained from commercial sources, were maintained in individual cages in an air-conditioned (74°F) room. In the first experiment, rabbits of known age from 21-day weanlings to 105-day young adults were injected intramuscularly with 0.05 μg/kg of body weight of the various cobamide analogues. Inorganic cobalt-60 was injected at a dose of 2.5 mμg/kg of body weight which is approximately the same level of cobalt present in the cobamide analogues. The animals were killed by cervical fracture after 24 hours and the liver and kidneys analyzed for radioactivity.

In the second experiment, young adult female rabbits weighing 2 to 2.5 kg were placed in metabolism cages and fed ad libitum or starved for 7 days before beginning the experiments. After obtaining a 24-hour control urine sample, the animals were injected intramuscularly on the eighth day with the various cobamide analogues and inorganic cobalt 60 at the same dose rate as in experiment 1. In all cases, the dosage given was based on the initial pre-starved weight. Urine samples were collected for three days, the animals killed on the eleventh day and the tissues analyzed for radioactivity as previously described in detail (Rosenthal, '59, '61a). Statistical evaluations were made by means of Student's *t* test. The cobamide analogues were Co^{00}-cyanocobalamin, (1 μc/μg); Co^{60}-hydroxocobalamin (0.4 μc/μg); and Co^{60}-5,6-dimethylbenzimidazolyl cobamide coenzyme (0.6 μc/μg).

RESULTS

Data obtained in the first experiment are illustrated in figure 1 and 2. The uptake of cobamide analogues and inorganic

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[2] Generous quantities of Co^{60}-cyanocobalamin and Co^{60}-hydroxocobalamin were supplied by Dr. E. Alpert, Merck Sharpe and Dohme, Inc., Rahway, New Jersey, and Co^{60}-5,6-dimethylbenzimidazolyl cobamide coenzyme by Dr. D. Perlman of Squibb and Company, New Brunswick, New Jersey.

[3] Samson, G. D., S. D. J. Yeh and B. F. Chow 1961 Urinary excretion of hydroxy-(1), cyanocobalamin (II) and 5,6-dimethybenzimidazole-B_{12} coenzyme (III) following intramuscular injection to man. Federation Proc., 20: 451 (abstract).

[4] Glass, G. B. J., D. H. Lee, H. R. Skeggs and J. L. Stanley 1962 Long acting effects of hydroxocobalamin on B_{12} deficiency in man. Federation Proc., 21: 471 (abstract).

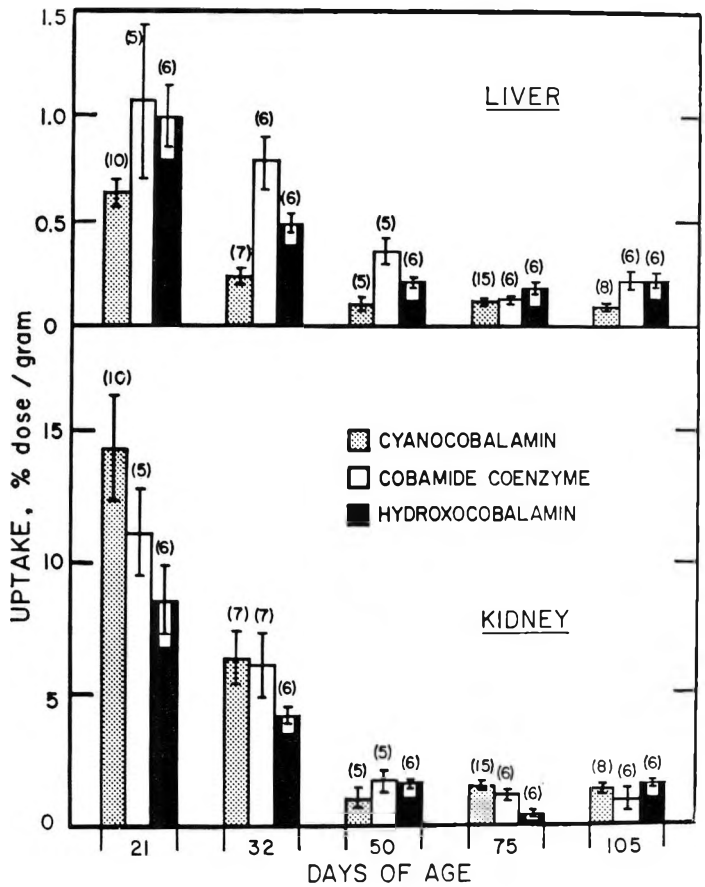

Fig. 1 The uptake of Co^{60}-labeled cobamide analogues in kidneys and liver from female rabbits of various ages 24 hours following intramuscular injection. The vertical bars represent standard errors of the mean and the number of animals studied is shown in parentheses. Note uptake units as compared with table 1.

cobalt in both liver and kidney tissue of 21-day-old rabbits is relatively high and decreases progressively to about 50 to 75 days of age, reaching young adult levels by this time. Kidney tissue of 21-day-old rabbits accumulated three to ten times more of the various substances than liver tissue, but this difference tended to disappear in older animals, although statistically significant values between kidney and liver uptakes were obtained with 105-day-old rabbits.

The pattern of uptake for the cobamide analogues differs between kidney and liver tissue (fig. 1). The uptake of cyanocobalamin was lower than hydroxocobalamin or cobamide coenzyme in liver tissue for all age groups studied but was most marked in young rabbits between 21 and 50 days of age. In contrast with liver tissue, kidney tissue accumulated more cyanocobalamin than hydroxocobalamin or cobamide coenzyme in 21-day-old rabbits, but the difference between the various analogues tended to disappear in the older age groups. Kidney tissue also accumulated greater amounts of inorganic cobalt than liver tissue.

Data obtained in the second experiment is shown in table 1. The uptake of all of the substances tested was significantly higher in the kidneys from starved rabbits. In fed rabbits, kidney tissue accumulated more of the injected dose of cyanoco-

balamin > hydroxocobalamin > coenzyme > cobalt. Except for the uptake of cyanocobalamin and hydroxocobalamin in kidney tissue of fed rabbits, which is not significantly different due to the large standard error of the mean values, all of the other values are significantly different from each other at $P = 0.05$ or less. In contrast with fed rabbits, the accumulation of the various cobamide analogues in the kidneys of starved rabbits was essentially similar. The uptake of inorganic cobalt was markedly less than the cobamide analogues and the uptake in kidneys of starved rabbits, although significantly greater than ad libitum-fed animals, was not nearly as marked.

The concentration of all of the injected cobamide analogues and inorganic cobalt was greatest in kidney and liver tissue and considerably lower in all other tissues studied. Although the livers of starved rabbits appeared to accumulate more of the cobamide analogues than ad libitum-fed rabbits, these differences may not be significant since the livers of starved rabbits are smaller thus resulting in an apparent increase in concentration. This relationship

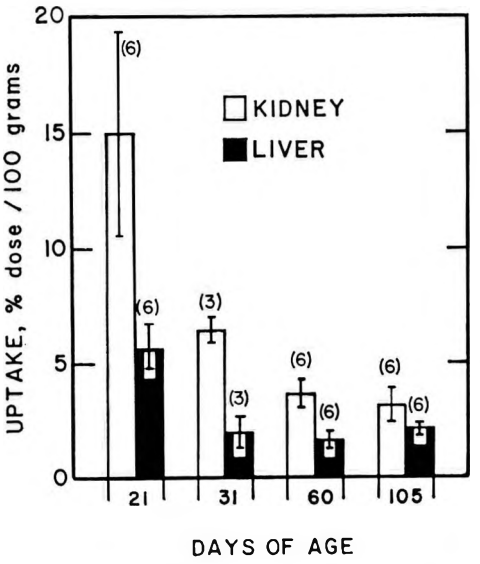

Fig. 2 The uptake of inorganic Co^{60} in kidneys and liver from female rabbits of various ages 24 hours following intramuscular injection. The symbols are the same as figure 1. Note uptake units as compared with figure 1.

TABLE 1

Tissue accumulation and urine excretion of cobamide analogues and inorganic cobalt in female rabbits three days after injection

Treatment	Kidney	Liver	Heart	Brain	Spleen	Muscle	Urine
			% dose/100 gm tissue ± SE				*% dose ± SE*
Cyanocobalamin							
Fed	130.7 ± 37.9[1](6)[2]	10.42 ± 1.87[1](6)	4.58 ± 0.50 (6)	1.27 ± 0.21 (6)	5.59 ± 1.00 (6)	1.70 ± 0.45 (6)	5.30 ± 1.05(6)
Starved	455.2 ± 42.9[1](5)	30.00 ± 5.58[1](5)	5.86 ± 0.97 (5)	1.99 ± 0.38 (5)	11.53 ± 5.15 (4)	1.55 ± 0.23 (5)	7.31 ± 1.44(5)
Hydroxocobalamin							
Fed	82.2 ± 15.0[1](6)	27.35 ± 4.99[1](6)	7.39 ± 0.79[1](6)	1.41 ± 0.11 (6)	12.46 ± 0.96[1](6)	2.20 ± 0.17[1](6)	3.77 ± 0.49(6)
Starved	416.9 ± 10.3[1](7)	39.07 ± 5.08[1](7)	4.91 ± 0.83[1](5)	1.12 ± 0.26 (5)	7.15 ± 1.14[1](5)	1.37 ± 0.21[1](5)	6.30 ± 1.18(7)
Coenzyme							
Fed	43.7 ± 13.9[1](6)	23.14 ± 2.22[3](6)	6.84 ± 0.61 (6)	1.70 ± 0.32 (6)	10.57 ± 2.03[3](6)	2.26 ± 0.45 (6)	3.61 ± 0.60(6)
Starved	367.1 ± 112.1[1](5)	51.72 ± 14.35[3](5)	6.74 ± 1.01 (5)	1.08 ± 0.72 (5)	5.82 ± 1.06[3](5)	2.04 ± 0.43 (5)	5.57 ± 2.06(5)
Cobalt							
Fed	3.29 ± 0.49[1](5)	1.70 ± 0.68 (5)	0.94 ± 0.26[3](5)	0.17 ± 0.04[3](5)	0.72 ± 0.12 (4)	0.38 ± 0.19 (5)	46.67 ± 4.50(4)
Starved	6.99 ± 0.97[1](4)	2.74 ± 0.42 (4)	1.49 ± 0.36[1](4)	0.37 ± 0.10[3](4)	1.96 ± 1.08 (4)	0.18 ± 0.05 (4)	41.83 ± 5.04(4)

[1] Significantly different at 1% level between fed and starved animals.
[2] Number of animals in parentheses.
[3] Significantly different at 5% level between fed and starved animals.

was previously discussed in detail (Rosenthal, '59, '61b). In general, heart, brain, spleen and muscle from starved or ad libitum-fed rabbits accumulated about the same amount of the various cobamide analogues and inorganic cobalt although some significant differences occur.

The low tissue values obtained for inorganic cobalt is largely due to the urinary excretion of 45% of the injected dose during the three-day experimental period. The urinary excretion of the three cobamide analogues in ad libitum-fed animals was lower and approximates 3 to 5% of the injected dose during the three-day experimental period. In starved animals, the urinary excretion of the cobamide analogues increased to 5 to 7%, an increase which is of doubtful significance.

The uptake of the cobamide analogues in tissues of 105-day-old rabbits killed either 24 hours (fig. 1) or three days (table 1) after injection was similar and indicates a slow turnover rate for these analogues, which is in accord with previous data obtained with cyanocobalamin (Rosenthal, '59).

DISCUSSION

The results of these experiments suggest that the starvation syndrome, resulting in greater accumulation of injected cobamide analogues, is a general but as yet ill-defined phenomenon. The incorporation of cyanocobalamin into kidney tissue of fed animals is greater than hydroxocobalamin or the coenzyme, but cyanocobalamin is incorporated to the least extent into liver tissue suggesting that different mechanisms may be operative for the various tissues. Previous *in vitro* experiments with kidney and liver slices demonstrated distinct metabolic differences for cyanocobalamin uptake for these two tissues (Jud and Rosenthal, '61). Although the incorporation of the injected cobamide analogues in the kidneys of rabbits fed ad libitum differ significantly from each other, the general similarity of the incorporation suggests that the various analogues are being bound on the same sites or that the analogues are rapidly converted to very similar substances. Toohey and Barker ('61) found the coenzyme form of the vitamin to predominate in the livers of many animals, including rabbit, but little is known concerning the metabolic fate of injected cobamide analogues.

The relatively high accumulation of injected cobamide analogues in the livers and kidneys of young animals, that rapidly decreases to adult levels, is of particular interest. This marked accumulation may reflect an increased requirement of vitamin B_{12} in young growing animals. Sadovsky et al. ('59) found serum vitamin B_{12} levels to be higher in newborn infants than in their mothers, again suggesting a greater demand for the vitamin by the rapidly developing fetus. This explanation may also be tenable for the inorganic cobalt since it is well known that cobalt has erythropoietic activity and that the rate of erythropoiesis in young animals is greater than in older animals (Underwood, '56).

The uptake of injected cyanocobalamin in the kidneys of ad libitum-fed rabbits, obtained in the present experiments, is greater than that previously found (Rosenthal, '59, '61b) but reasons for these divergent results are unknown. In reports of studies performed between 1955 to 1958 (Rosenthal, '59) and 1958 to 1960 (Rosenthal, '61a), the uptake of injected cyanocobalamin in rabbit kidneys ranged from 0.14 to 0.35% of the dose/gm of tissue. These values were obtained on animals procured from different breeders, for animals maintained with a variety of commercial rabbit pellets and for animals maintained in air-conditioned or non-air-conditioned rooms, during summer or winter and in either Rochester or St. Louis. However, since the latter part of 1961, the accumulation of the injected dose in kidneys has increased to 1.31% of the dose/gm of tissue, as shown in this report, and has been constant during this time. On the other hand, the uptake of injected cyanocobalamin in liver, heart, brain, spleen, and muscle and the urinary excretion of the injected vitamin has been essentially similar during the past years.

This variability may be due to genetic or hereditary factors, changes in physiological state of the animal or to environmental conditions beyond our control. The use of commercial rabbit food, composed largely of naturally occurring nutrients may also introduce a factor of uncer-

tainty. Unfortunately, attempts to substitute highly purified, semi-synthetic diets for commercial rabbit chow have not been successful. Although many other factors may be related to this variability, clarification of the phenomenon must await further study.

SUMMARY

The uptake of intramuscularly injected cyanocobalamin, hydroxocobalamin, 5,6-dimethylbenzimidazolyl cobamide coenzyme and inorganic cobalt by liver and kidneys was relatively high in 21-day-old female rabbits, decreasing to constant levels between 50 and 105 days of age. Kidney tissue accumulated more of the injected dose than liver tissue for all of the substances tested. In rabbits subjected to starvation for 10 days, the accumulation of the injected cobamide analogues in kidney tissue was markedly increased above that of fed animals. All other tissues studied showed little, if any, change between fed or starved rabbits. These data suggest a fundamental but ill-defined relationship between growth, starvation and vitamin B_{12} metabolism that results in increased accumulation of injected cobamide analogues into kidney tissue of rabbits.

ACKNOWLEDGMENT

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A Striking Difference in the Thiamine-sparing Action of Fat on Deficiencies Produced by Oxythiamine and Pyrithiamine[1]

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It has been reported by van Eys ('61) that glycerophosphate dehydrogenase contains a special prosthetic group which appears to be an oxidation product of thiamine and has been named thiamic acid by its discoverer. It occurred to us that it was possible that this prosthetic group might also be necessary for the reverse reaction, that is, the reduction of dihydroxyacetone phosphate to the corresponding glycerol derivative. If this were true, an inadequate intake of thiamine might lead to a deficiency of glycerol. Furthermore, it could be that at least part of the thiamine-sparing action of fats, first reported many years ago by Evans and Lepkovsky ('29), might be due to the glycerol moiety of the fats. Although we soon found that liberal amounts of glycerol did not give rats any protection against a dietary deficiency of thiamine, these experiments led to a further study of the thiamine-sparing action of fat; especially with deficiencies produced by the thiamine inhibitors, oxythiamine and pyrithiamine.

EXPERIMENTAL

Young female rats obtained from a commercial supplier[2] served throughout as experimental animals. The diets used were of the usual purified type, and the composition of the low-fat (no. 1) and high-fat (no. 2) diets are given in table 1. The diet high in fat contained 35% by weight of lard, and the other constituents, except sucrose, were increased so that on a caloric basis this ration contained the same amount of protein, salts, vitamins, and other ingredients as the low-fat diet. The thiamine inhibitors, when used, were also increased correspondingly in the diets high in fat.

The data are presented in a series of growth curves which show the number of days that each group of animals was on the experimental regimen as well as the average changes in weights of the animals. A curve depicting the average change in weight of a group of surviving animals may fluctuate erratically as the animals die. In an attempt to lessen this fluctuation, each curve was drawn as a solid line up to the day on which the first death occurred. The average weight at death was subsequently taken for all the animals in any one group and this value was marked on the chart on the day the last animal died. This point was then connected with the end of the solid line by a broken line. Thus, all of the animals died in the period represented by the broken line.

In the first experiment, 32 rats were divided into 8 groups of 4 animals each. One group received the complete low-fat diet containing 5 mg of thiamine/kg. Another was given the corresponding high-fat diet (7.0 mg of thiamine). The animals of both of these groups grew normally, with the animals fed the high-fat diet gaining a little more weight (131 gm gain) than those supplied with the low-fat intake (122 gm). The curves for these animals are not given. The animals of these two groups were discontinued after receiving the respective diets for 43 days.

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[1] A preliminary report of this work has been presented. Jones, J. H. and L. K. Govardhan 1961 The sparing action of fat on thiamine. Federation Proc., 20: 448 (abstract).

[2] Carworth Farms, Inc.

Three of the groups were fed diets containing no added thiamine. One of these (curve A, fig. 1) received the low-fat basal diet with no supplement, another (curve B) received the same diet with 38 gm of glycerol/kg replacing an equal weight of sucrose, and the remaining group (curve C) was given the high-fat diet. The amount of glycerol given (curve B) is approximately equal to that in 350 gm of lard. The glycerol gave no protection under these conditions. Generally it has been assumed that fat spares thiamine but does not replace it. Here, however, the fat had a definite, although small, effect on both growth and length of life even though the ration contained no added thiamine.

Since the effect of fat, and possibly glycerol, might be enhanced by a small amount of thiamine, three other groups

TABLE 1

Composition of diets

Ingredients	No. 1 Low-fat diet		No. 2 High-fat diet	
	gm/kg	*gm/1,000 Cal.*	*gm/kg*	*gm/1,000 Cal.*
Casein, vitamin-free	180	46.7	259	46.7
Salt mixture[1]	40	10.4	58	10.4
Cellulose	20	5.2	29	5.2
Corn oil[2]	20	5.2	29	5.2
Lard	0	0	350	63.0
Sucrose	738	192.0	272	49.0
Choline chloride	2	0.5	3	0.5

Vitamins	Low-fat diet	High-fat diet
	mg/kg	*mg/kg*
Thiamine chloride·HCl	variable	variable
Riboflavin	5.0	7.0
Pyridoxine·HCl	5.0	7.0
Ca pantothenate	30.0	42.0
Nicotinic acid	30.0	42.0
Pteroylglutamic acid	1.0	1.4
Biotin	0.2	0.28
Inositol	30.0	42.0
2-Methyl-1,4-naphthoquinone	1.0	1.4
Three drops of oleum percomorphum given once a week		

[1] Jones and Foster ('42).
[2] Mazola, Corn Products Company, Argo, Illinois.

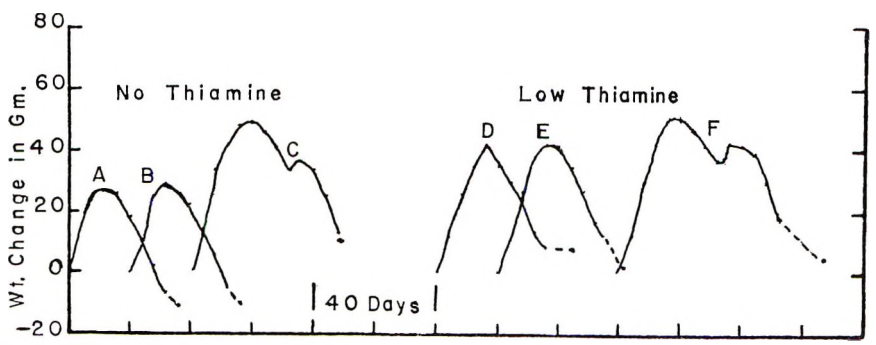

Fig. 1 Effect of glycerol and of fat on a dietary deficiency of thiamine; 4 animals in each group. Curve A shows changes in weight of animals fed diet 1 with no supplement. Curve B, diet contained 38 gm of glycerol/kg of diet. Curve C, animals given high-fat diet. Animals represented by curves D and E received 0.2 mg of thiamine, and curve F 0.28 mg of thiamine/kg of diet. Otherwise, these animals were given the same diet as those in curves A, B, and C respectively.

were fed the same diets as above except that 0.2 (curve D), 0.2 (curve E) and 0.28 mg (curve F) of thiamine were added to the three diets, respectively. This small amount of thiamine slightly increased the length of life and growth of the first two groups but had no discernible effect on the animals fed the high-fat ration. Here again the glycerol gave no protection against the deficiency. It is possible that the effect of the fat could have been augmented by still higher levels of thiamine, for Evans and Lepkowsky ('29) and Salmon and Goodman ('37), with rats, and Arnold and Elvehjem ('39), with pups, reported much more striking results from fat even though no thiamine was added to the basal diets. These experiments were made, however, before the use of highly purified diets had been made possible. Furthermore, a direct comparison is not feasible because of differences in the amount of fat, and in some cases the type of fat, used.

THIAMINE INHIBITORS

To reduce the amount of inhibitor necessary to produce a deficiency, only 1.25 mg of thiamine were supplied/kg of the low-fat diet (no. 1) and 1.75 mg/kg of the high-fat diet (no. 2). That is, the high-fat ration contained more thiamine and inhibitor on a weight basis than did the low-fat diet but an equal amount on a caloric basis. In each experiment the ratio by weight of inhibitor to vitamin was the same in the low-fat and high-fat diets. The above levels of thiamine produced good growth when no inhibitor was added (curves E and F, fig. 2). At these levels of thiamine, the animals consuming the high-fat diet gained about 40 gm more than the animals fed the low-fat diet.

Oxythiamine. Four experiments were conducted with oxythiamine. In two of these, the ratio of inhibitor to vitamin was 40/1, and in the other two experiments it was 80/1. Especially at the higher level of oxythiamine, diarrhea was quite prevalent among the animals fed the low-fat diet, but was not observed in the animals receiving the diet high in fat. Increasing the thiamine to 10 mg/kg of the low-fat, high-oxythiamine diet prevented the diarrhea but did not stimulate growth so well as did the high level of fat. The results of one of the experiments with the higher ratio are summarized in figure 2. The

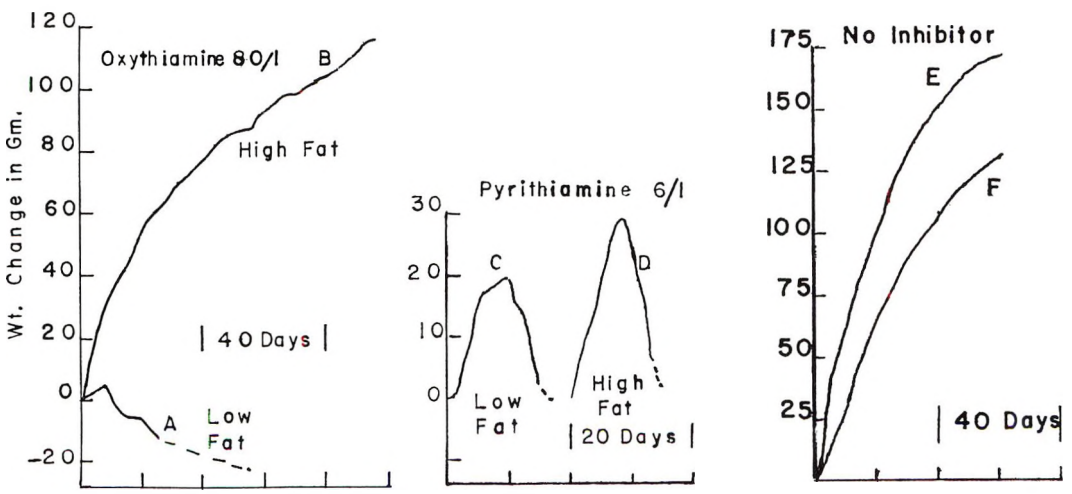

Fig. 2 Effect of fat on a deficiency of thiamine produced by oxythiamine or pyrithiamine. Four animals in group represented by curve B and 5 animals in each of other five groups. Animals fed low-fat diets received 1.25 mg of thiamine and those fed high-fat diets, 1.75 mg of thiamine/kg of diet. Curve A, low-fat diet plus oxythiamine at a ratio of 80/1 of thiamine; curve B, oxythiamine plus high-fat diet; same ratio. Curve C, low-fat diet plus pyrithiamine at ratio of 6/1. Curve D, high-fat diet plus pyrithiamine at 6/1 ratio with thiamine. Curve E, high-fat diet, no inhibitor. Curve F, low-fat diet, no inhibitor.

animals (5 in number) fed the low-fat diet (curve A) did poorly from the start and all were dead by the 55th day. Diarrhea was noted occasionally which was the only abnormal sign observed other than anorexia with loss of weight followed by death. The animals[3] fed the high-fat diet showed no deficiency signs and their overall growth rate approached that on the ordinary, complete, purified diet. The experiment was terminated after 98 days.

The other experiments with oxythiamine gave comparable results. It was noted, however, that with the low-fat diet containing oxythiamine, rats with an initial weight of 45 to 50 gm responded to oxythiamine more severely than those with starting weights of 55 to 70 gm. In the first experiment using a 40/1 ratio the average initial weight was 58 gm, and two of five animals fed the low-fat diet, although in poor condition, were still alive when the experiment was terminated at 74 days. In the second experiment the average starting weight was 44 gm and all animals were dead by the twenty-ninth day. In the two experiments using the inhibitor at an 80/1 ratio, the average initial weights were 68 gm for the heavier rats and 50 gm for the lighter animals, and the last animal in the former group died on the fifty-fifth day as compared with the thirty-first day for the latter group. Except in the one case noted in footnote 3, there were no deaths among the animals receiving oxythiamine and the diet high in fat.

Pyrithiamine. Pyrithiamine[4] was incorporated into the low-fat and high-fat diets at 20/1, 10/1, 8/1, 6/1, 4/1 and 2/1 inhibitor to thiamine ratios. The results with the first 4 ratios were almost identical. For the first few days the animals gained weight rapidly, with those fed the high-fat diet gaining a little more than those receiving the diet low in fat. The animals then reversed themselves, lost weight rapidly and died about the fifteenth day. Without exception and about two days before death, all of these animals developed extreme polyneuritic signs. This was in marked contrast with the animals receiving oxythiamine, as none of them showed any such symptoms. This difference between oxythiamine and pyrithiamine was first observed by Cerecedo et al. ('51).

The results obtained at the 4/1 and 2/1 ratios were essentially the same as those above except the succession of events developed more slowly, with the animals receiving the latter ratio living about 30 days before death occurred. Usually the animals receiving the lard lived, on an average, from one to four days longer than those fed the low-fat ration. Curves C and D of figure 2 show the results obtained at the 6 to 1 ratio.

As the increased gain in weight by the animals fed the diet high in fat was completely consistent, the question arose as to the mechanism involved. Was the fat partially counteracting the effect of the inhibitor directly or was the effect due simply to the higher caloric content of the high-fat diet? In an attempt to answer this question, an experiment was conducted using three groups of animals. One group was given the usual low-fat diet (1.25 mg of thiamine/kg of diet) and two groups the diet high in fat (1.75 mg of thiamine). One of the latter two was pair-fed with the first group, but the pairings were done on a caloric basis instead of the usual weight basis. The other group was given an unrestricted supply of the diet high in fat. All animals were given pyrithiamine at a ratio of 2/1 with the thiamine. The results of this experiment are summarized in figure 3.

When one of a pair of animals being pair-fed dies, the question arises as to what to do with the remaining animal especially if it is the control. Under the conditions existing in this type of experiment the deficient animal usually stops eating a few days immediately preceding death. This was the case in these experiments and when this situation developed, the paired control continued to be fed a starvation diet until death. Of the 6 pairs one of the controls died before its paired mate, one died on the same day as its mate, and with the other 4 pairs the ani-

[3] Originally this group included 5 rats, but one did poorly from the start and died on the 56th day. As this was the only animal receiving oxythiamine and the high-fat diet which failed to remain in a relatively good state of nutrition is was eliminated from further consideration.

[4] Kindly furnished by Dr. Ashten Cuckler, Merck Institute for Therapeutic Research, Rahway, N. J.

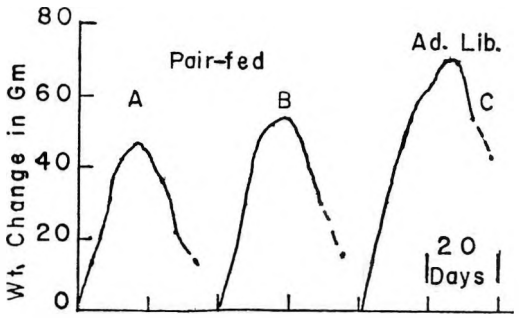

Fig. 3 Effect of high-fat diet pair-fed and given *ad libitum* on a deficiency of thiamine produced with pyrithiamine. Six animals in each group. Curve A, low-fat diet plus pyrithiamine at 2/1 ratio with thiamine. Curve B, high-fat diet plus pyrithiamine (2/1 ratio) pair-fed on caloric basis with group A. Curve C, high-fat diet plus pyrithiamine (2/1 ratio) fed ad libitum.

mal receiving the low-fat intake died before its paired control.

The average number of days the animals had received the diet at the time of death was 30 and 33 for the paired animals fed the low- and high-fat diets, respectively, and 34 days for the unrestricted high fat animals. All animals, however, showed definite polyneuritic symptoms before death.

These three groups of animals (fig. 3, curves A, B and C) gained a maximum of 47, 55 and 73 gm, respectively.

This experiment was repeated in its entirety with 6 animals again in each group. The animals lived an average of 27, 28, and 34 days and gained a maximum of 47, 45 and 67 gm, respectively, for the groups fed the low-fat diet, pair-fed diet and the unrestricted high-fat diet. All of the animals of the first and third groups showed marked polyneuritic signs, but in two of the second group (paired, high-fat diet) these symptoms were not observed. These animals probably died of starvation.

The pair-fed animals receiving the high-fat diet obtained a maximum gain in weight of 8 gm more than the low-fat animals in one experiment and 2 gm less in the other, compared with 26 and 20 gm for the full-fed, high-fat animals. In neither case did the diet high in fat prevent the development of the polyneuritic signs nor extend life by more than a few days. The pair-fed, high-fat animals resembled more closely the low-fat animals than the unrestricted high-fat rats with respect to growth and length of life. This suggests that the slight differences between the low-fat and high-fat diets were probably due to the higher caloric content of the latter and not due to a specific effect on either the vitamin or inhibitor.

DISCUSSION

From the data presented here, a high level of fat in the diet exerts a pronounced protective action against a deficiency of thiamine produced by oxythiamine, a metabolic inhibitor of thiamine. In contrast, fat has practically no effect on a deficiency resulting from the administration of pyrithiamine which likewise is an inhibitor of thiamine. The observations give additional support to the concept that these two compounds inhibit different metabolic reactions or enzyme systems requiring thiamine. It is possible that oxythiamine inhibits some reaction which can be bypassed by fat or its metabolites; that is, with a high-carbohydrate diet the organism as a whole may be dependent upon some reaction requiring thiamine and inhibited by oxythiamine. On the other hand, it may be possible for the organism to substitute the metabolism of fat for carbohydrate and that the particular reaction being inhibited by oxythiamine is not essential to the metabolism of the fat. Consequently, the organism can function in a manner nearly normal when sufficient fat is available. This does not appear to be true with pyrithiamine. In this case either the organism cannot substitute fat for carbohydrate, in the reaction being blocked, or fat also requires thiamine for this particular step.

SUMMARY

Thirty-five per cent of lard in a synthetic diet to which no thiamine was added gave a definite but slight protective action against the thiamine deficiency. The effect of the fat was not increased by the addition of 0.2 mg of thiamine/kg of diet. The incorporation of 3.8% of glycerol afforded no protection against the thiamine deficiency. The above amount of lard exerted a pronounced "sparing action" when a thiamine deficiency was produced by oxythiamine but gave only a slight protection

against a thiamine deficiency caused by pyrithiamine, and this appeared to be due more to the increased caloric intake than to an effect on thiamine or pyrithiamine.

ACKNOWLEDGMENT

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Interrelationships of Cold Exposure and Amino Acid Imbalances[1]

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From the recent work concerning the effects of amino acid imbalances, it is obvious that the main obstacle in studying these phenomena is the refusal of animals to consume voluntarily adequate amounts of the imbalanced diet which, in turn, causes a major difficulty in interpreting and extrapolating the experimental data. In an effort to overcome this problem and thus to approach the normal physiological state of the animal, such techniques as forced-feeding (Deshpande et al., '58), spaced-feeding (Kumta et al., '58) or insulin injections (Spolter and Harper, '61) have been used as means of inducing a higher food intake. However, these techniques proved either unsatisfactory or even detrimental to the animal and the results obtained may be subjected to an alternative explanation.

In view of the finding in this laboratory (Vaughan and Vaughan, '57, '59, '60, '61) that appetite of cold exposed rats, while characteristically depressed by vitamin deficiencies, was simultaneously stimulated by the low environmental temperature, it seemed to us advantageous to use cold exposure as a tool to induce voluntarily a higher intake of imblanced diets in studying amino acid imbalances in the rat. The results of such experiments are presented in this work.

EXPERIMENTAL

Male, Sprague-Dawley rats, ranging in weight from 160 to 200 gm were used in all experiments. In each experiment they were divided into two groups. One group was placed in a cold room held at 7°C, while the other group remained in an animal room at 25°C. All rats were housed in individual wire cages and both the diets and water were offered on an ad libitum basis. The animals and refused food were weighed every second day.

The basal low-protein fibrin or casein diets and the imbalancing amino acid mixture were patterned after those used by Kumta and Harper ('60). The basal diet consisted of 6% of fibrin, 85% sucrose (or 9% of crude casein and 82% of sucrose), 5% of corn oil, and 4% USP salt mixture no. 2. The vitamin mixture supplied 2,000 units of vitamin A, 222 units of vitamin D, 11.1 mg of α- tocopherol and the following in mg: ascorbic acid, 100; inositol, 11.1; choline chloride, 166.5; menadione, 5; *p*-aminobenzoic acid, 11.1; niacin 10; pyridoxine hydrochloride 2.22; riboflavin 2.22; thiamine hydrochloride 2.22; Ca pantothenate, 40.3; also 44 μg of biotin, 200 μg of folic acid and 3 μg of vitamin B_{12}/100 gm of diet.

The imbalancing amino acid mixture consisted of: (in per cent) DL-methionine, 0.4; DL-phenylalanine, 0.6; L-leucine, 0.4; DL-isoleucine, 0.4; DL-valine, 0.7; L-lysine·HCl, 0.6; L-arginine·HCl, 0.2; L-tryptophan, 0.2; DL-threonine, 0.4; L-glutamic acid, 1.0; and L-histidine·HCl, 0.4. The imbalanced diet was created by adding one or two amino acids, or a mixture of amino acids to the basal diets. All substitutions were made at the expense of sucrose in the basal diet. At the end of 21 days the animals were decapitated and the blood collected in centrifuge tubes containing a drop of heparin solution. The liver was immediately excised, chilled in chipped ice and assayed for arginase, glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT).

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[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association. (AFR 160-94).

Arginase determination was carried out according to the procedure of Brown and Cohen ('59) with the following modifications: 10% liver homogenates were prepared in glass double-distilled water and 0.2 ml of the 1:1250 water-diluted homogenate was added to the incubation mixture consisting of 0.2 ml of L-arginine 0.85 M, pH 9.5; 0.2 ml of glycine 0.50 M, pH 9.5; 0.2 ml $MnCl_2$, 0.005 M and of 1.2 ml of glass double-distilled water. Incubations were carried out in 10-ml beakers at 38°C with shaking for 30 minutes. Reactions were stopped with 2 ml of 0.5 M perchloric acid and after centrifugation the clear supernatant was analyzed for urea with 1-phenyl-1:2-propanedione-2-oxime according to the method of Archibald ('44) as modified by Ratner ('55). To obtain the value for endogenous urea, the enzyme in the incubation mixture was inactivated with perchloric acid and the incubation was carried out as usual. The value for endogenous urea was subtracted from the experimental urea value. Both the incubations and the urea determinations were carried out on at least duplicate samples.

The GOT and GPT were measured by following the oxidation of DPNH with a Beckman DK recording spectrophotometer according to procedures similar to those used by Wróblewski and LaDue ('56) and LaDue et al. ('54). Aliquots of tissues were analyzed for nitrogen content by acid digestion and the nesslerization procedure. Since there was no difference in the nitrogen content of the livers, the results of the enzyme assays were calculated as micromoles of products per minute per 100 grams of body weight. It was felt that this method of expressing the activity more closely reflects the animals' total ability to produce a given product in relation to their needs.

The levels of plasma and urinary amino nitrogen were determined spectrophotometrically by the ninhydrin method. Since urea interferes with the color development, it was separated from the amino acids in the deproteinized plasma or the urine by paper chromatography in a *n*-butanol:95% ethanol: water (4:1:1) system. The amino acids were eluted with water and the color developed according to the procedure of Saifer et al. ('60). Individual urine collections were carried out in standard metabolism cages over a one-day period and the urine samples were kept frozen until analyzed.

RESULTS AND DISCUSSION

The design and results of the first experiment are presented in table 1. The data show that an addition of 5% of L-leucine to the casein basal diet caused a severe retardation of growth and a depression in food consumption both in the warm and the cold groups. Despite a highly toxic level of leucine in the diet, the cold-exposed animals increased their food consumption almost 75% over the corresponding warm group, thus also proportionally increasing the absolute daily intake of this amino acid from approximately 395 mg to 690 mg. However, such an increment in leucine intake had no additional adverse effect on growth.

TABLE 1

Effect of cold exposure and amino acid imbalance on growth and food consumption of rats (exp. 1)

Group	Diet	Δ Weight		Food intake	
		Warm rats	Cold rats	Warm rats	Cold rats
		gm/3 weeks	*gm/3 weeks*	*gm/day*	*gm/day*
1	9% Casein	61.1 ± 2.4[1]	50.8 ± 6.3	11.9 ± 0.39	17.3 ± 0.23
2	9% Casein + 5% L-leucine [7]	−7.1 ± 3.0	−3.9 ± 3.2	7.9 ± 0.32	13.8 ± 0.15
3	6% Fibrin	42.8 ± 2.8	39.6 ± 3.8	11.7 ± 0.45	17.1 ± 0.15
4	6% Fibrin + amino acid mixture minus histidine	22.7 ± 2.7	39.0 ± 2.7	8.6 ± 0.28	16.7 ± 0.31

[1] Standard error of the mean for 7 rats.

A similar effect of cold on food consumption and growth can be seen in group 4, in which an amino acid imbalance was induced by adding an amino acid mixture lacking histidine to the fibrin basal diet. This imbalanced diet caused a considerable decrease both in food intake and the rate of growth of the warm rats, thus confirming similar findings of Kumta and Harper ('60).

In contrast, the cold-exposed animals readily consumed the imbalanced diet and, consequently, grew as well as the controls (group 3). However, in another experiment (table 3, group 7) the histidine-lacking diet did not cause any growth depression when offered to the rats maintained in the warm environment, although a marked growth response was again obtained in the cold group. Thus, it appears that an omission of histidine from the amino acid mixture used produces only a borderline imbalance which can be readily corrected by cold exposure.

An imbalance produced by omitting isoleucine from the amino acid mixture was studied in the second experiment (table 2, group 2). Again, the lack of isoleucine produced a pronounced growth retardation and a depression of food consumption in the group kept warm, an effect reported previously by Kumta and Harper ('60). However, these adverse effects were overcome to a great extent by the cold exposure. The higher food intake of the cold-exposed animals is reflected in the higher levels of the amino acid nitrogen both in the plasma and in the urine.

Incorporation of 0.4% of DL-methionine and 0.6% of DL-phenylalanine in the fibrin diet (group 3) produced a severe imbalance in the warm group which is in agreement with the findings of Deshpande et al. ('58). On the other hand, these two amino acids failed to produce any retarding effect on growth and food consumption in the cold-exposed animals, despite the fact that their absolute intake was increased by 90%. Rats force-fed a similar diet by Desphande et al. ('58) died within two or three days, apparently due to their inability to metabolize efficiently the diet. In contrast, the cold-exposed animals not only tolerated a higher intake of the two imbalancing amino acids, but grew as well as the controls.

TABLE 2

Effect of cold exposure and amino acid imbalance on growth, food consumption and urinary and plasma amino nitrogen of rats (exp. 2)

Group	Diet	Δ Weight		Food intake		Urinary NH_2-N		Plasma NH_2-N	
		Warm rats	Cold rats	Warm rats	Cold rats	Warm rats	Cold rats	Warm rats	Cold rats
		gm/3 weeks	*gm/3 weeks*	*gm/day*	*gm/day*	*mg/24 hours*	*mg/24 hours*	*mg/100 ml*	*mg/100 ml*
1	6% Fibrin	42.4 ± 1.5[1]	50.8 ± 5.8	12.2 ± 0.58	19.0 ± 0.72	3.99 ± 0.21	8.89 ± 0.25[2]	7.81 ± 0.32	10.77[2] ± 0.46
2	6% Fibrin + amino acid mixture minus DL-isoleucine	19.3 ± 3.0	36.7 ± 4.3	9.5 ± 0.32	17.6 ± 0.67	18.98 ± 0.36[3]	25.78 ± 0.41[3]	9.34[3] ± 0.36	10.68 ± 0.27
3	6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	28.4 ± 4.6	58.4 ± 3.5	10.4 ± 0.42	19.8 ± 0.14	10.88 ± 0.62[3]	16.12 ± 0.57[3]	8.44 ± 0.29	10.26 ± 0.35

[1] Standard error of the mean for 7 rats.
[2] Difference from warm group same diet ($P < 0.05$).
[3] Difference from fibrin group ($P < 0.05$).

The leucine-isoleucine antagonism, as defined and reported by Harper et al. ('55) and Benton et al. ('56) was studied in the third experiment (table 3). When either isoleucine (group 2) or isoleucine and valine (group 4) were omitted from the amino acid mixture, a severe growth depression occurred in the two warm groups. Furthermore, an omission of leucine together with isoleucine (group 3) or leucine together with isoleucine and valine (group 5) restored normal growth, showing that an excess of leucine is responsible for some metabolic alteration affecting food intake, which in turn leads to a depression in growth. This phenomenon was completely overcome by the cold exposure. The cold-exposed animals were able to increase food intake and performed as well as either of the controls (group 1). As expected, the complete amino acid mixture added to the fibrin basal (group 6) supported an excellent rate of gain.

The activity of the two transaminases and of the arginase was higher in the cold controls than in the warm controls, possibly as a result of an increased food intake in the cold. An omission from the amino acid mixture of either isoleucine alone (group 2), or isoleucine plus leucine (group 3) or isoleucine plus valine (group 4) resulted in a uniform increase in the activity of all three enzymes. The enzyme activity in this case was not, however, further affected by the environmental temperature.

Although the basic mechanism concerning the phenomena presented herein remains to be determined, the results of these experiments indicate that a moderate cold stress is an effective agent in correcting and overcoming amino acid imbalances. Maintained in a warm environment, a rat suffers a severe metabolic disorder when it consumes a diet containing an imbalanced amino acid mixture. However, it can effectively metabolize and utilize it for tissue synthesis when exposed to cold. The data of Kumta et al. ('58) indicate that feeding an imbalanced diet either increases the breakdown of tissue proteins or that an imbalanced diet is not properly utilized for the protein synthesis. From the data presented in the present work it appears that the cold-exposed animals are able to use an imbalanced diet for the formation of the tissue proteins. The cold exposed animals could possibly accomplish this step by catabolizing preferentially the imbalancing portion of the amino acid mixture and utilizing it for heat production. The remaining balanced portion of the mixture could be then effectively utilized for the protein synthesis.

SUMMARY

Moderate cold exposure was used as a tool in inducing a higher food intake in experiments dealing with amino acid imbalances in the rat. The data indicate that the rats kept at 25°C suffer a severe metabolic disorder after ingesting amino acid imbalanced diets. In contrast, the animals kept at 7°C readily consumed the imbalanced diets and, consequently, grew as well as the controls. Liver glutamic-oxalacetic and glutamic-pyruvic transaminase and liver arginase activity were increased as a result of a cold exposure. It is suggested that a moderate cold stress is an effective agent in correcting and overcoming amino acid imbalances.

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TABLE 3

Effect of cold exposure and the leucine-isoleucine antagonism on growth, food consumption and glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and arginase activities (exp. 3)

Group		1	2	3	4	5	6	7
Diet		6% Fibrin	6% Fibrin + amino acid mix minus isoleucine	6% Fibrin + amino acid mix minus isoleucine, minus leucine	6% Fibrin + amino acid mix minus isoleucine, minus valine	6% Fibrin + amino acid mix minus isoleucine, minus leucine, minus valine	6% Fibrin + complete amino acid mix	6% Fibrin + amino acid mix minus histidine
Δ Weight, gm/3 weeks	Warm	35.8 ±1.4[1]	18.7 ±3.3	37.2 ±2.2	18.4 ±2.6	32.8±6.6	85.7±5.4	42.6±2.8
	Cold	41.3 ±5.2	34.5 ±6.0	44.5 ±4.3	35.6 ±3.0	39.3±7.0	76.3±5.0	58.8±4.8
Food intake, gm/24 hours	Warm	13.4 ±0.38	11.7 ±0.52	12.8 ±0.54	11.7 ±0.46	14.5±0.87	15.1±0.36	14.0±0.60
	Cold	18.2 ±0.53	18.0 ±0.79	18.9 ±0.64	17.7 ±0.59	19.3±0.57	20.8±0.22	19.8±0.39
GOT, μmole/min/ 100 gm body weight	Warm	510 ±54	718[2] ±35	788[2] ±55	698[2] ±66			
	Cold	753[3] ±54	856 ±57	814 ±73	829 ±86			
GPT, μmole/min/ 100 gm body weight	Warm	129 ±9.7	194[2] ±10.3	195[2] ± 9.3	191[2] ±20.2			
	Cold	171[3] ±8.0	229 ±30.2	196 ±17.9	191 ±18.5			
Arginase, μmole/min/ 100 gm body weight	Warm	526 ±29	692[2] ±36	721[2] ±42	678[2] ±29			
	Cold	652[3] ±38	718 ±45	695 ±45	701 ±31			

[1] Standard error of the mean for 7 rats.

[2] Difference from fibrin group ($P < 0.05$).

[3] Difference from warm group — same diet ($P < 0.05$).

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